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TRASET 201

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicant	:	Przybylski et al.
Filed	:	August 9, 2004
Serial No.	:	10/686,249
Publication No.	:	US 2006/0135752 A1
For:	:	METHOD OF OBTAINING BIOLOGICALLY ACTIVE COLLAGEN FROM SKINS OF THE SALMONIDAE FISH

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August 16, 2006

Commissioner for Patents
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(3) A copy of each listed patent or publication in written form or at least the pertinent portions; and

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(4) An English language translation of all the necessary and pertinent parts of any non-English language patent or publication in written form relied upon.

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(6) Return Postcard

Documents submitted:

U.S. Publication No. 2003/0004315
U.S. Patent No. 5420248 (+ Cover Sheet WO93/01241)
U.S. Publication No. 2003/0032601 (+ Cover Sheet WO01/64046)
European Patent No. EP 0 602 297
French Patent No. FR 2 783 836 (+ Translation)
French Patent No. FR 2806415 (+ Translation)
Polish Patent No. PL 291055 (+ Translation)
Polish Patent No. PL 312122 (+ Translation)

Respectfully submitted,

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- -

CERTIFICATE OF SERVICE

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Steven M. Hoffberg
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US005420248A

United States Patent [19]

Devictor et al.

[11] **Patent Number: 5,420,248**

[45] **Date of Patent: May 30, 1995**

[54] **UNPIGMENTED FISH SKIN, PARTICULARLY FROM FLAT FISH, AS A NOVEL INDUSTRIAL SOURCE OF COLLAGEN, EXTRACTION METHOD, COLLAGEN AND BIOMATERIAL THEREBY OBTAINED**

[75] Inventors: **Pierre Devictor**, Lyons; **Roland Allard**, St.Genis-Laval; **Eric Perrier**, Vienne; **Alain Huc**, Sainte Foy-les-Lyon, all of France

[73] Assignee: **Coletica**, Lyons, France

[21] Appl. No.: **170,353**

[22] PCT Filed: **Jul. 2, 1992**

[86] PCT No.: **PCT/FR92/00621**

§ 371 Date: **Jan. 4, 1994**

§ 102(e) Date: **Jan. 4, 1994**

[87] PCT Pub. No.: **WO93/01241**

PCT Pub. Date: **Jan. 21, 1993**

[30] **Foreign Application Priority Data**

Jul. 4, 1991 [FR] France 91 08400

[51] **Int. Cl.^{6}** **C07K 14/78**; C07K 1/14; A61K 38/39; A61K 7/00

[52] **U.S. Cl.** **530/356**; 530/857; 602/42; 602/43; 602/48; 602/50; 106/124; 106/125; 424/443; 424/444; 424/445

[58] **Field of Search** 530/356, 857; 602/42, 602/43, 48, 50; 106/124, 125; 424/443, 444, 445, 572, 574

[56] **References Cited**

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Primary Examiner—Michael G. Wityshyn
Assistant Examiner—Nancy J. Degen
Attorney, Agent, or Firm—Millen, White, Zelano & Branigan

[57] **ABSTRACT**

The present invention relates to the use of unpigmented skin from flat fish as novel industrial source of collagen. As unpigmented skin, the ventral skin is used in particular, from sole, dab, turbot, brill. Native acid-soluble collagen is advantageously extracted and separated by precipitation from the supernatant. The invention makes it possible to improve the collagen yield at reduced cost while preserving the native properties of the protein, and in a reproducible manner.

18 Claims, No Drawings

UNPIGMENTED FISH SKIN, PARTICULARLY FROM FLAT FISH, AS A NOVEL INDUSTRIAL SOURCE OF COLLAGEN, EXTRACTION METHOD, COLLAGEN AND BIOMATERIAL THEREBY OBTAINED

The present invention relates essentially to the use of unpigmented fish skin, particularly from flat fish, as novel industrial source of collagen, to a method for extracting the collagen, as well as to the collagen and biomaterial thereby obtained.

BACKGROUND OF THE INVENTION

Collagen, as a protein for strengthening the connective tissues of human beings, finds ever expanding applications in the cosmetic and pharmaceutical fields.

The main properties are as follows:

good mechanical properties,
action on cell development, hence on tissue regeneration,
hemostatic power,
excellent biocompatibility,
biodegradability,
natural support improving bioavailability towards numerous active agents.

Owing to its remarkable qualities, said protein has contributed to the production of numerous biomaterials for which the market is continuously expanding.

Among the many collagen-based preparations, the main ones seem to be as follows:

solution for cosmetics,
solution for making artificial skin,
hemostatic sponge,
healing dressings,
bone-reconstruction material,
protection capsules against active agents and improvement of their bioavailability,
biomaterials in solid or liquid form for filling flabby tissues.

When reading the foregoing list, which is in no way exhaustive, it is easy to realize that collagen is bound to become a substance of great industrial importance.

Collagen materials will be developed all that quicker as their cost price reduces.

This is the reason why the inventors have tried to use new sources of collagen with a view to implementing the industrial methods, improving yields, hence reducing the costs of preparation of the protein.

At present, the tissue mainly used for obtaining collagen is calf skin.

But two difficulties are met when using this base material.

Firstly, the skin is not, in general, removed in perfectly hygienic conditions, which implies having to use the skin as soon as possible after the animal is slaughtered.

Secondly, the skin must undergo an important treatment for removing the hairs and subcutaneous tissues. Such treatment is carried out in tanning installations implying the construction of a workshop independent from the site where the collagen is transformed into biomaterials. This type of infrastructure requires relatively high investments which will reflect on the cost of the obtained collagen.

Moreover, although calf skin is a tissue from a very young animal, it furnishes relatively small quantities of acid-soluble collagen, about 12 g of dry protein for 1 kg of fresh skin.

In order to avoid as much as possible having to treat the skin, and to improve extraction yields, the inventors have had the idea of using unpigmented fish skin.

The inventors have indeed found that pigmented fish skin is not suitable because the pigment is very difficult to eliminate during the collagen preparation process. The protein obtained in this case leads to the production of impure pigmented biomaterials.

The inventors have therefore turned their attention to fish whose skin is unpigmented and available in large quantities. The species falling in that catagory are flat fish, particularly those fished on an industrial basis such as sole, dab, turbot, brill, which are subjected to a filleting or cutting operation. The ventral skin of these fish is unpigmented, it is available, scaled, in large quantities.

SUMMARY OF THE INVENTION

Therefore, in a first aspect, the present invention relates to the use of unpigmented fish skin as novel industrial source of collagen. Preferably, said unpigmented skin is from a flat fish, particularly a ventral skin. Advantageously, said unpigmented skin is obtained after the filleting or cutting of the fresh fish and frozen immediately after filling/cutting, thus guaranteeing a very good quality of the base material, both from the bacteriological standpoint and from the standpoint of the native property of the protein.

Moreover, an unexpected advantage resides in the fact that the skin such as recovered and frozen can be used fight away for collagen extraction without any prior tanning treatments. It has also been found that the quantity of collagen extracted from said base material is about three times greater than that obtained from calf skin.

Consequently, the use of this novel source of collagen will lead to obtaining a protein of reduced cost with reproducibly preserved native properties.

In a second aspect, the present invention further relates to a method for extracting native collagen, characterized in that it uses unpigmented fish skin from which collagen is extracted.

According to a variant embodiment of this method, the unpigmented skin is the skin of a flat fish, particularly a ventral skin. Advantageously, said skin is obtained after filleting or cutting fresh fish which is frozen immediately after filleting/cutting.

Said skin is also used for extracting collagen without any prior tanning treatment.

According to a particular variant of embodiment, the skin used is unpigmented skin from flat fish fished on an industrial basis, such as for example sole, dab, turbot, brill.

According to a particular variant of embodiment, native acid-soluble collagen is extracted by extraction in an acid solution, as known to the man skilled in the art. Then, the native collagen is separated by precipitation from the supernatant, by addition of a precipitating agent, such as sodium chloride. Anyone who wishes may effect a decrosslinking of said native collagen.

The invention also relates to the native collagen obtained from unpigmented fish skin, and to any biomaterial produced totally or partially with such a collagen.

The invention will now be described with reference to an example of collagen extraction using the extraction method according to the invention, given solely by way of illustration and which could not possibly limit the scope of the invention.

EXAMPLE

Preparation of acid-soluble collagen from 40 kg of frozen sole skins

1. Grinding of the skin

The ventral skins of sole fished industrially are supplied in frozen blocks of 20 kg. These are cut into blocks of 2 kg with a band saw. The obtained pieces are then ground and defrosted with a disk mill equipped with a 40 l sealed tank. 39 kg of ground material are obtained.

2. Washing of the ground material

The ground material is washed in two successive baths of phosphate buffer (21.7 g/l NA2 HPO4 and 0.78 g/l of KH2 PO4). The ground material concentration being 1 kg for 5 l of bath and the contact time 1 hour. The ground material is separated from the buffer by centrifuging using a continuous centrifuge turning at 4000 revs/min.

The phosphate is then removed in the same conditions by two sucessive washes in deionized sterilized water.

3. Acid extraction

The ground material obtained is then placed in a 0.25M solution of acetic acid at a concentration of 1 kg for 10 l of bath. The mixture is stirred for 15 mins. and the supernatant is recovered by continuous centrifuging in the same conditions as hereinabove.

This extracting operation is carried out twice. The supernatants are collected together and the collagen that they contain is precipitated by addition of sodium chloride at a concentration of 7%. The obtained fibers are dialyzed against the deionized water until complete elimination of the salts. The obtained collagen can be either placed in acid solution again or lyophilized.

1.6 kg of dry collagen are obtained from 40 kg of fresh sole skins. The extraction yield is more than three times greater than that obtained with calf skin.

Here are the characteristics of a solution of acid-soluble collagen from sole skin in a medium at 0.1% sorbic acid.

Chemical analyses	
Dry materials	0.46
Mineral materials	0.08
Total nitrogen	0.075
Total Proteins (from nitrogen)	0.40
Hydroxyproline	0.03
Collagen from hydroxyproline	0.40
Acidity (in sorbic acid)	0.10
Physical analyses	
Electrophoresis	
subunits β(200,000 daltons) 56%	
α(100,000 daltons) 44%	
no subunits less than 100,000 daltons	
Programmed differential scanning calorimetry:	
start-of-denaturation temperature in °C.: 28	
end-of-denaturation temperature in °C.: 37	
enthalpy of denaturation (J/mg collagen): 5×10^{-2}	
X-ray diffraction	
presence of the helical structure.	

The amino acid composition of the acid-soluble collagen obtained in the preceding example and which was extracted from the ventral skin of sole, has also been established.

AMINO ACID COMPOSITION

Amino acids	Number of residues for 1,000 total residues
Cysteic acid	0.00
Hydroxyproline	59.58
Aspartic acid	56.74
Threonine	20.76
Serine	27.87
Glutamic acid	85.11
Proline	59.58
Glycine	326.64
Alanine	126.09
Cystine	0.00
Valine	27.59
Methionine	2.27
Isoleucine	12.68
Leucine	29.60
Tyrosine	0.00
Phenylalanine	15.47
Histidine	8.96
Hydroxylysine	4.15
Lysine	34.34
Tryptophane	0.00
Arginine	46.81

We claim:

1. A process for the extraction of native collagen, comprising providing a starting material consisting essentially of non-pigmented fish skin, extracting collagen from said non-pigmented fish skin, and recovering said collagen.

2. The process of claim 1, wherein said non-pigmented fish skin is a flat fish skin.

3. The process of claim 1, wherein said non-pigmented fish skin is a ventral skin of a flat fish.

4. The process of claim 2, wherein said non-pigmented fish skin is a ventral skin of a flat fish.

5. The process of claim 1, wherein said skin is obtained by cutting the skin from fresh fish and immediately freezing the skin after cutting.

6. The process of claim 1, wherein said fish is selected from the group consisting of sole, dab, turbot, and brill.

7. The process of claim 2, wherein said fish is selected from the group consisting of sole, dab, turbot, and brill.

8. The process of claim 1, comprising grinding the non-pigmented fish skin, placing the resultant ground fish skin in an acid solution to extract acid-soluble collagen, separating a supernatant containing said acid-soluble collagen, and recovering said acid-soluble collagen in native form by precipitation from said supernatant.

9. The process of claim 6, comprising grinding the non-pigmented fish skin, placing the resultant ground fish skin in an acid solution to extract acid-soluble collagen, separating a supernatant containing said acid-soluble collagen, and recovering said acid-soluble collagen in native form by precipitation from said supernatant.

10. The process of claim 8, further comprising intermediate steps after grinding said non-pigmented fish skin but prior to said acid extraction, of washing the ground skin with a phosphate buffer solution, separating said phosphate buffer solution from said ground skin, and washing the ground material with water to eliminate residual phosphate.

11. The process of claim 8, wherein said precipitation of a native collagen is performed by introducing said supernatant containing acid-soluble collagen into a sodium chloride solution to precipitate native collagen fibers.

12. The process of claim 10, wherein said precipitation of a native collagen is performed by introducing

said supernatant containing acid-soluble collagen into a sodium chloride solution to precipitate native collagen fibers.

13. Th process of claim 11, further comprising subjecting the resultant collagen fibers to dialysis against deionized water to eliminate salts so as to recover a native collagen essentially free of salts.

14. The process of claim 13, further comprising lyophilizing the resultant native collagen essentially free of salts.

15. The process of claim 14, wherein said native collagen has 56% of subunits β of 200,000 dalton, 44% of subunits α of 100,000 dalton, and does not have subunits lower than 100,000 dalton, as measured by electrophoresis and has a helicoidal structure.

16. The process of claim 14, further comprising shaping the resultant native collagen into a biomaterial.

17. Th process of claim 15, further comprising shaping the resultant native collagen into a biomaterial.

18. A process for the extraction of nativ collagen, comprising providing a starting material consisting essentially of non-pigmented flat fish skin, extracting collagen from said non-pigmented flat fish skin, and recovering said collagen, thereby obtaining a substantially collagen-depleted, non-pigmented fish skin, wherein said starting material is obtained by separating pigmented fish skin from non-pigmented fish skin.

* * * * *

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Bureau international

DEMANDE INTERNATIONALE PUBLIEE EN VERTU DU TRAITE DE COOPERATION EN MATIERE DE BREVETS (PCT)

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(74) Mandataires: PORTAL, Gérard etc. ; Cabinet Beau de Loménie, 55, rue d'Amsterdam, F-75008 Paris (FR).

(81) Etats désignés: CA, CS, HU, JP, KR, US, brevet européen (AT, BE, CH, DE, DK, ES, FR, GB, GR, IT, LU, MC, NL, SE).

Publiée
Avec rapport de recherche internationale.

(54) Title: USE OF UNPIGMENTED FISH SKIN, PARTICULARLY FROM FLAT FISH, AS A NOVEL INDUSTRIAL SOURCE OF COLLAGEN, EXTRACTION METHOD, AND COLLAGEN AND BIOMATERIAL THEREBY OBTAINED

(54) Titre: UTILISATION DE LA PEAU NON PIGMENTEE DE POISSON, EN PARTICULIER DE POISSON PLAT COMME NOUVELLE SOURCE INDUSTRIELLE DE COLLAGENE, PROCEDE D'EXTRACTION, COLLAGENE ET BIOMATERIAU AINSI OBTENUS

(57) Abstract

The use of unpigmented flat fish skin as a novel industrial source of collagen is described. In particular, the unpigmented skin used is the ventral skin, preferably of sole, dab, turbot and brill. Advantageously, native acid-soluble collagen is extracted and separated by precipitation from the supernatant. The collagen yield may thereby be reproducibly improved at reduced cost and without affecting the native properties of the protein.

(57) Abrégé

La présente invention concerne l'utilisation de la peau non pigmentée de poisson plat comme nouvelle source industrielle de la collagène. Comme peau non pigmentée, on utilise en particulier de la peau ventrale, de préférence à partir de sole, de limande, de turbot, de barbue. On extrait avantageusement du collagène acido-soluble à l'état natif que l'on sépare par précipitation à partir du surnageant. L'invention permet d'améliorer le rendement d'obtention de collagène avec un coût moins élevé tout en conservant les propriétés natives de la protéine, et de façon reproductible.

ZA ZGODNOŚĆ
mgr inż. Jacek Czabajski
Rzecznik Patentowy

US 20030004315A1

(19) **United States**

(12) **Patent Application Publication** (10) **Pub. No.: US 2003/0004315 A1**

Macdonald et al. (43) **Pub. Date: Jan. 2, 2003**

(54) COLLAGEN

(76) Inventors: **Grant Arthur Macdonald**, Nelson (NZ); **Kathleen Hofman**, Nelson (NZ)

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(21) Appl. No.: **10/129,129**

(22) PCT Filed: **Nov. 29, 2000**

(86) PCT No.: **PCT/NZ00/00237**

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Nov. 29, 1999 (NZ) 501386

Publication Classification

(51) **Int. Cl.^{7}** **C07K 14/78**

(52) **U.S. Cl.** **530/356**; 106/160.1

(57) ABSTRACT

The present invention relates to novel collagen products, methods for producing same, and to a novel test for assessing native collagen content of a protein solution. Particular collagen products are extracted from the skin of cold water fish. The collagen products comprise a high proportion of native collagen and are suitable for use as fining agents in the brewing and wine industries.

FIGURE 1

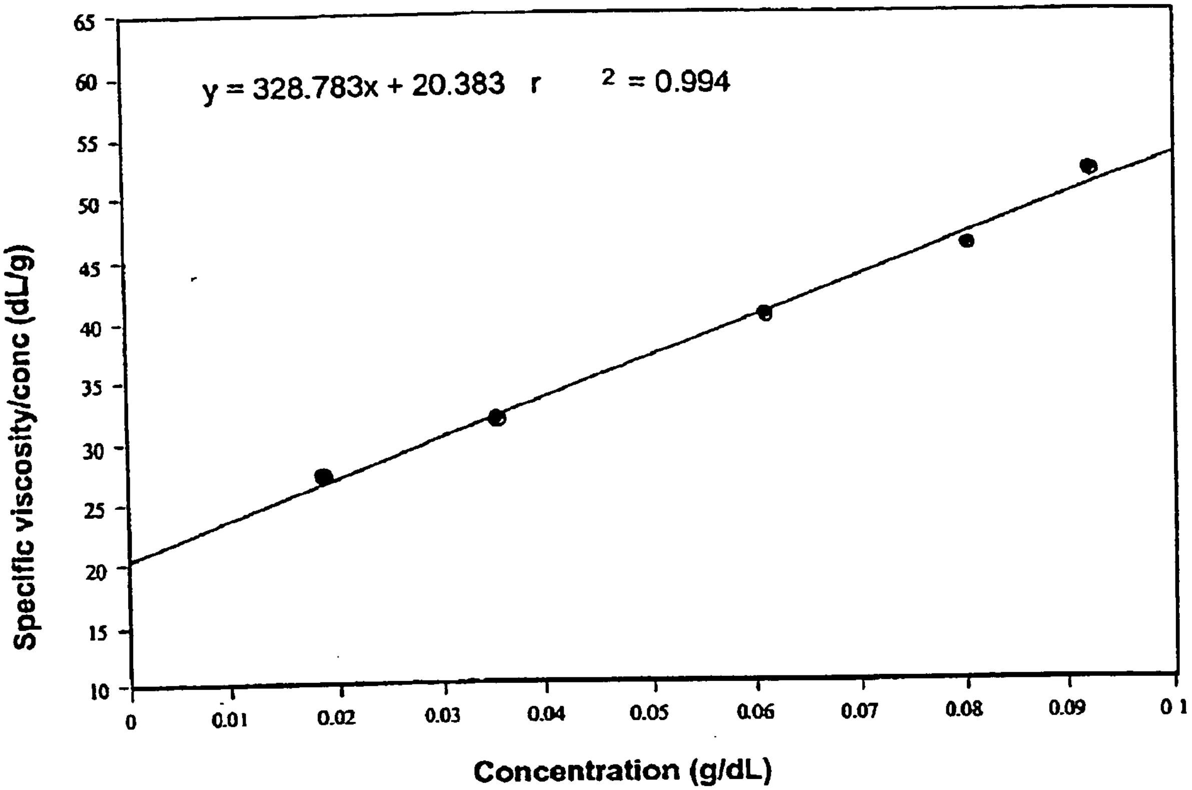

y = 328.783x + 20.383 r 2 = 0.994
Specific viscosity/conc (dL/g)
65
60
55
50
45
40
35
30
25
20
15
10
0
0.01
0.02
0.03
0.04
0.05
0.06
0.07
0.08
0.09
0 1
Concentration (g/dL)

FIGURE 2

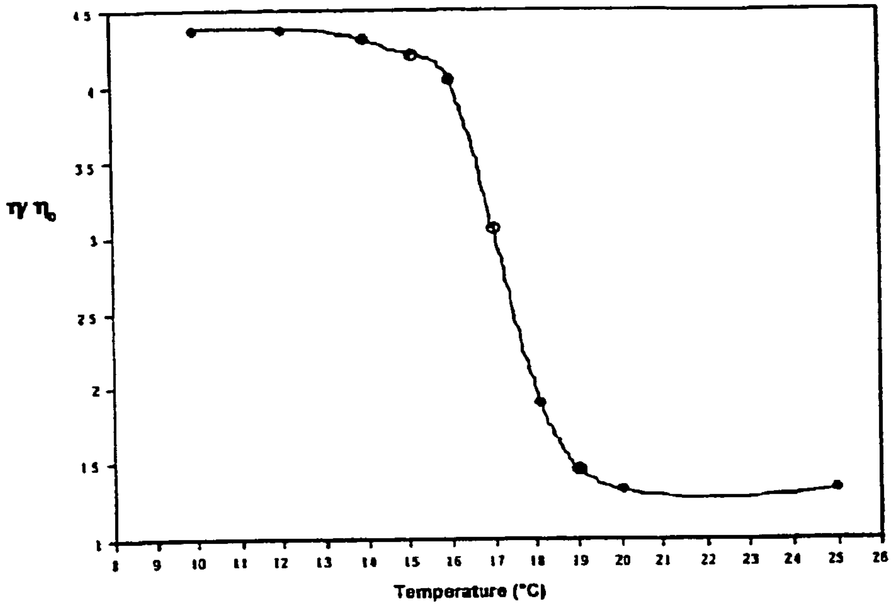


FIGURE 3

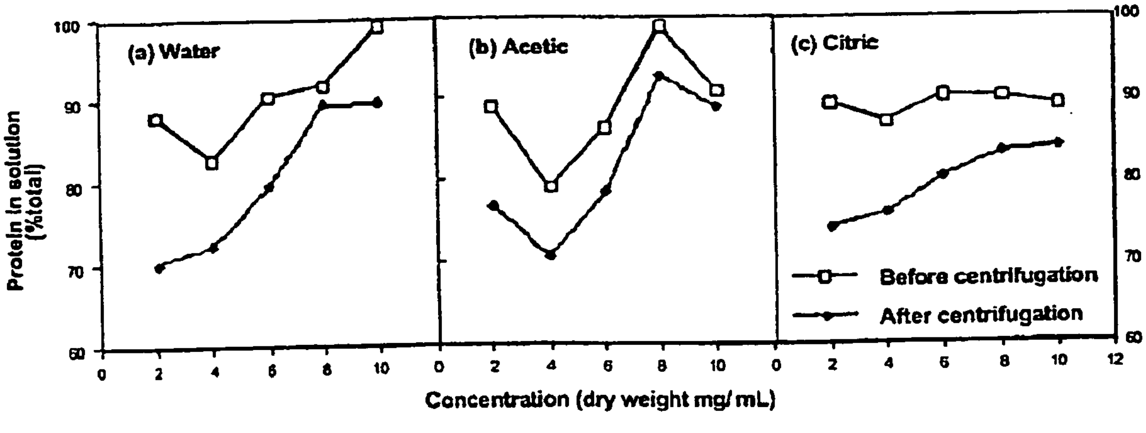


FIGURE 4a

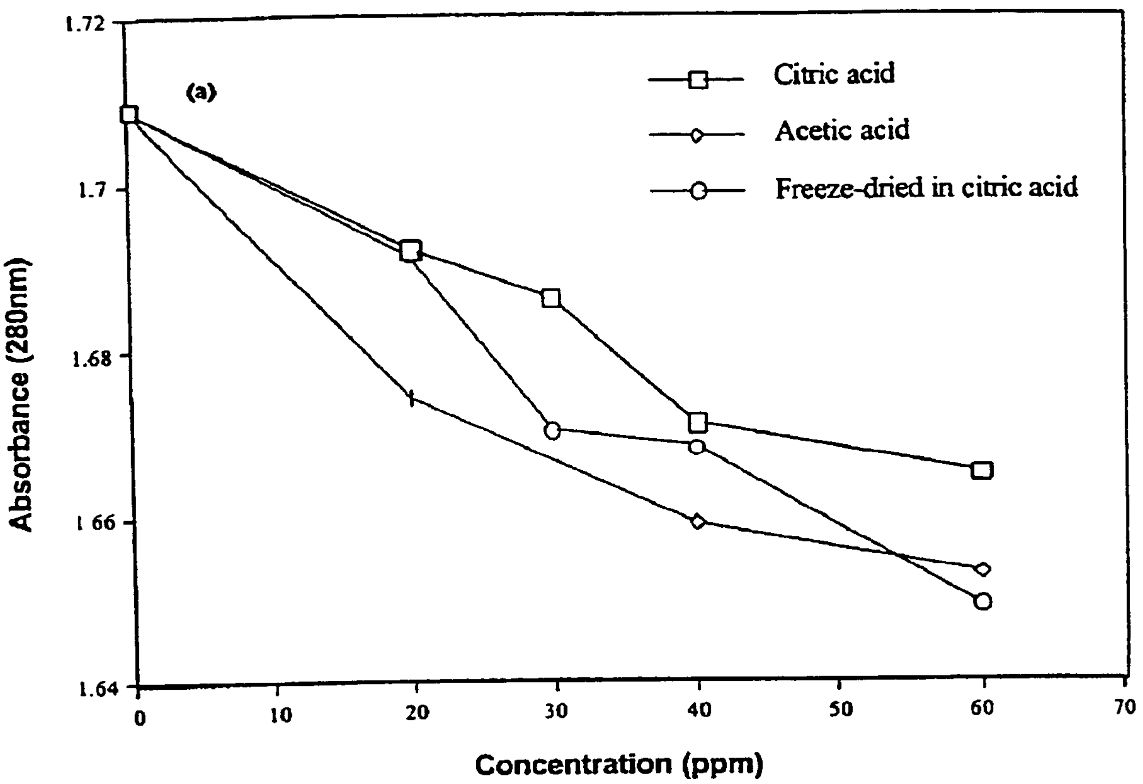
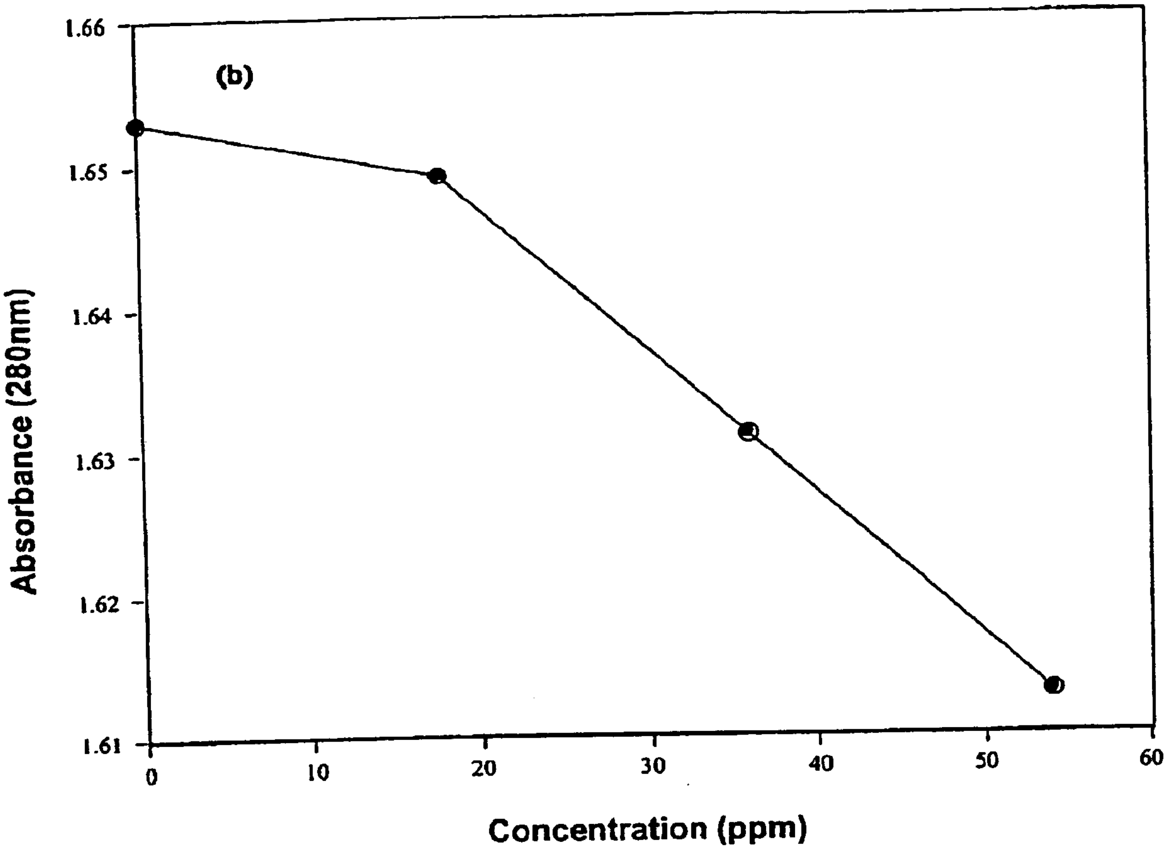

(a)
Citric acid
Acetic acid
Freeze-dried in citric acid
Absorbance (280nm)
1.72
1.7
1.68
1.66
1.64
0
10
20
30
40
50
60
70
Concentration (ppm)

FIGURE 4b


(b)
1.66
1.65
1.64
1.63
1.62
1.61
Absorbance (280nm)
0
10
20
30
40
50
60
Concentration (ppm)

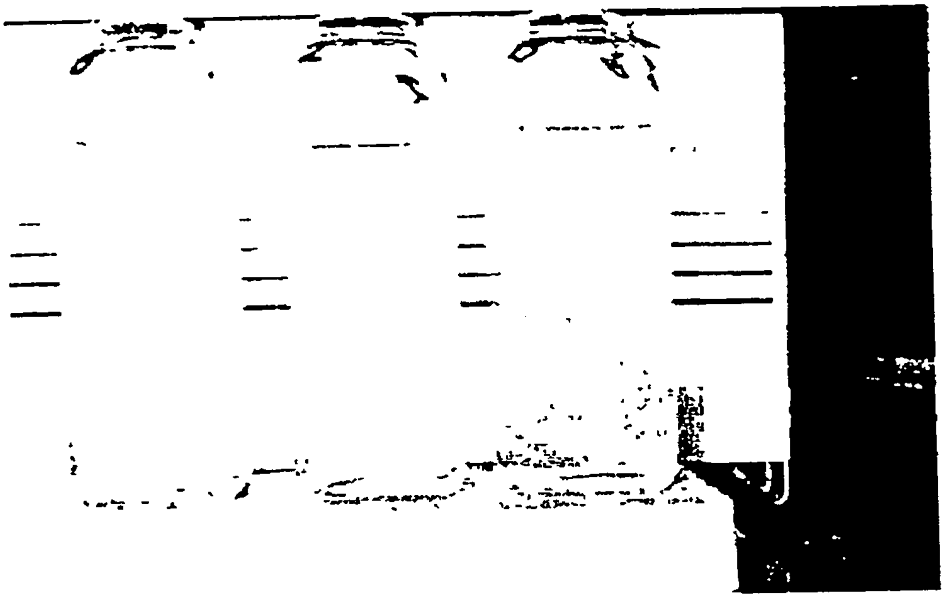


FIGURE 5

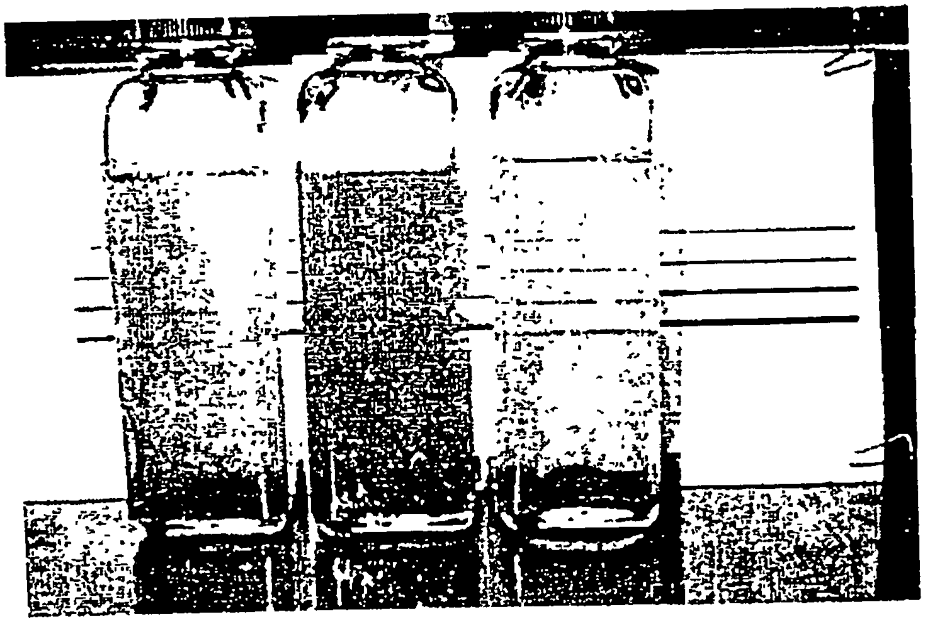


FIGURE 6

FIGURE 7

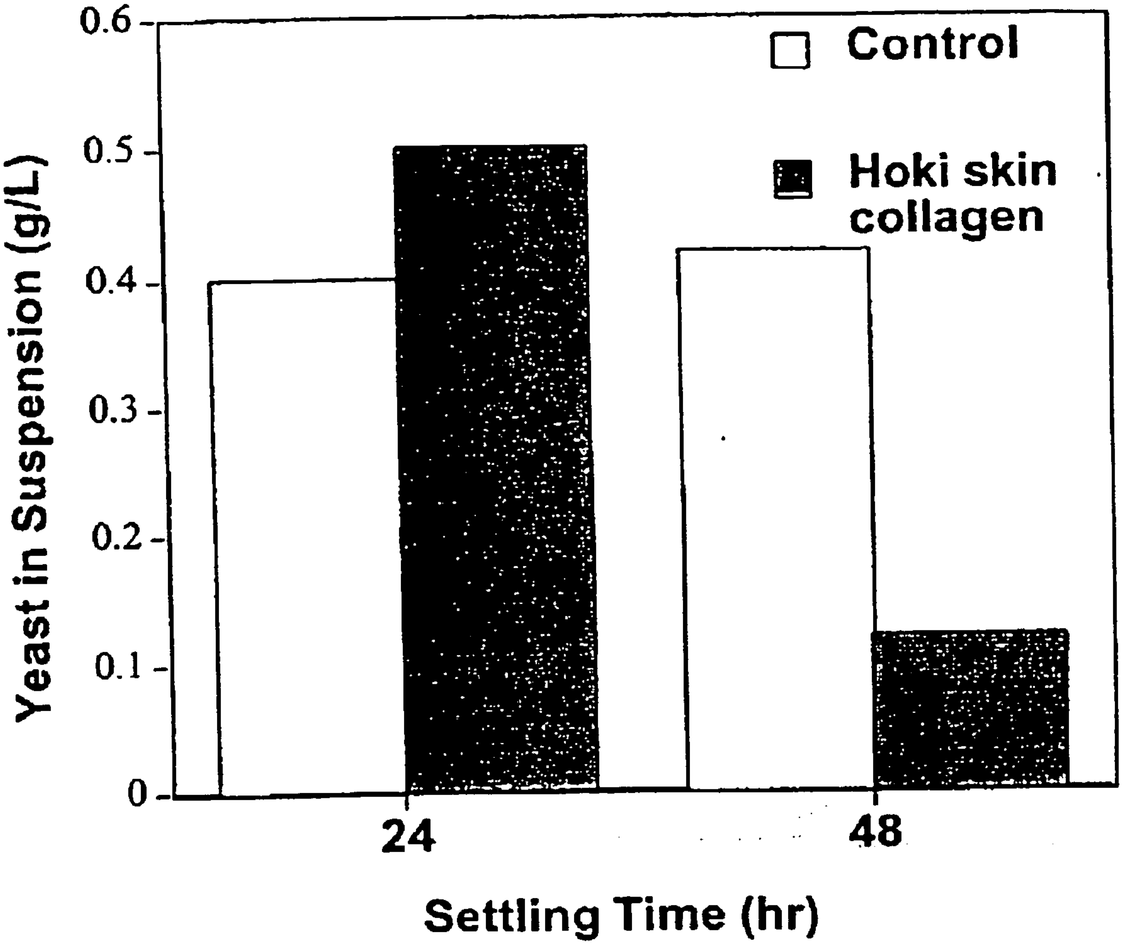

0.6
0.5
0.4
0.3
0.2
0.1
0
Yeast in Suspension (g/L)
☐ Control
■ Hoki skin collagen
24
48
Settling Time (hr)

FIGURE 8

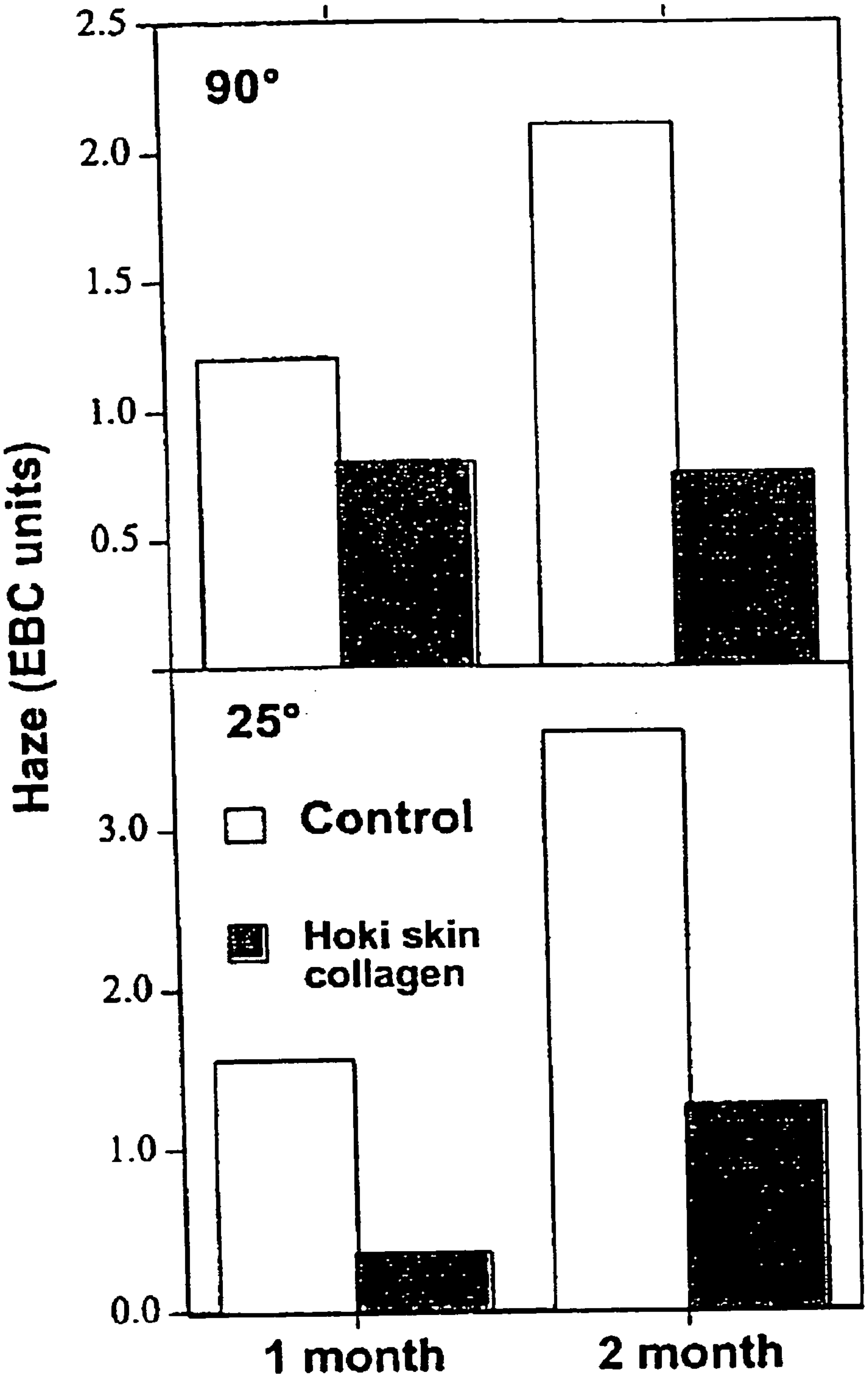

2.5
2.0
1.5
1.0
0.5
90°
25°
3.0
2.0
1.0
0.0
Haze (EBC units)
□ Control
▨ Hoki skin collagen
1 month
2 month

FIGURE 9

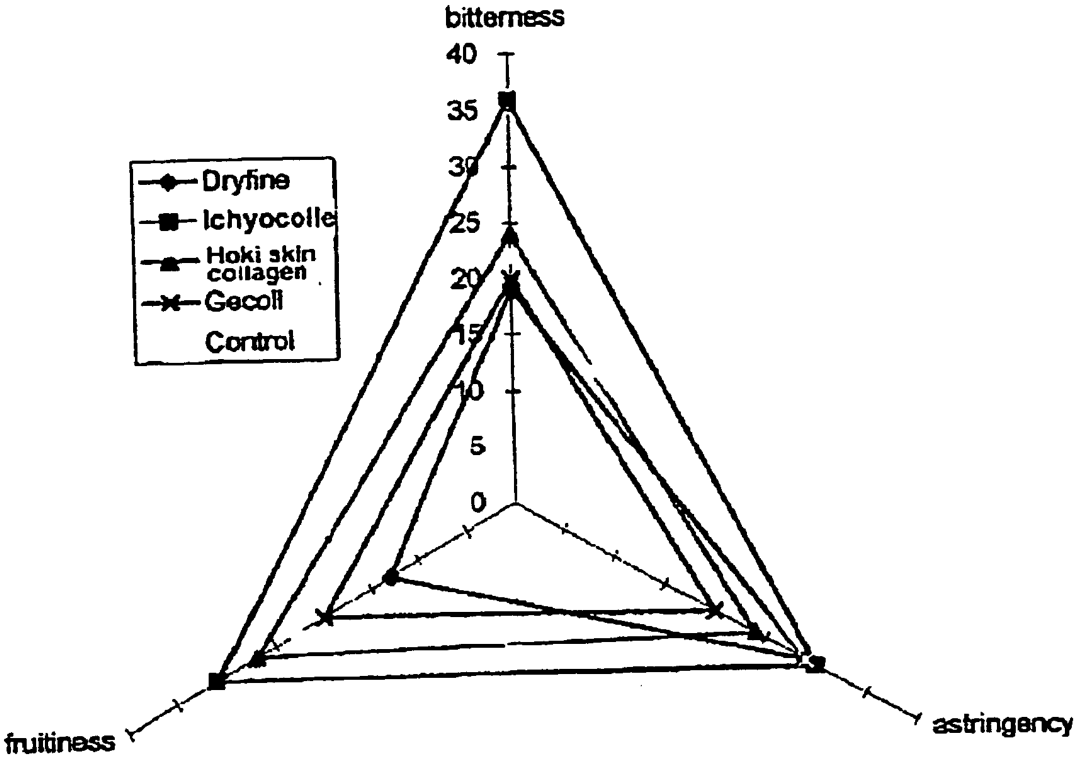

bitterness
40
35
30
25
20
15
10
5
0
astringency
fruitiness
Dryfine
Ichyocolle
Hoki skin collagen
Gecoll
Control

FIGURE 10

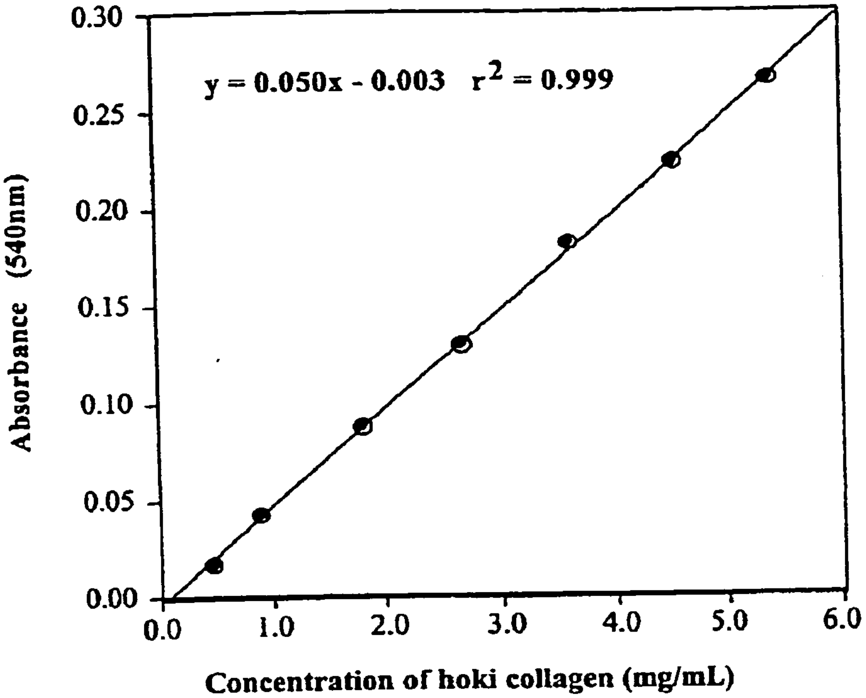


FIGURE 11

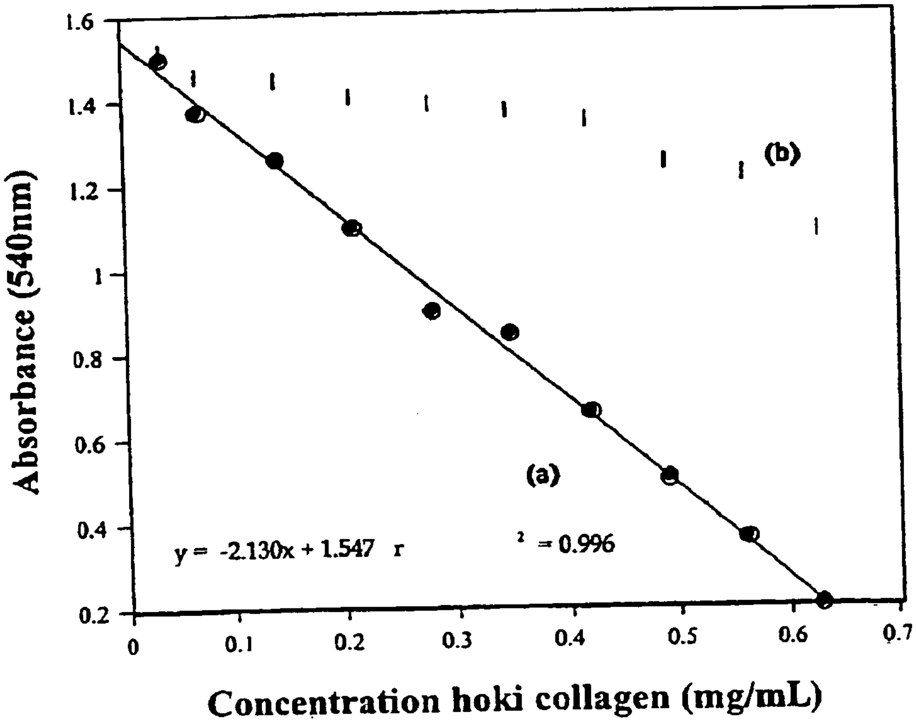
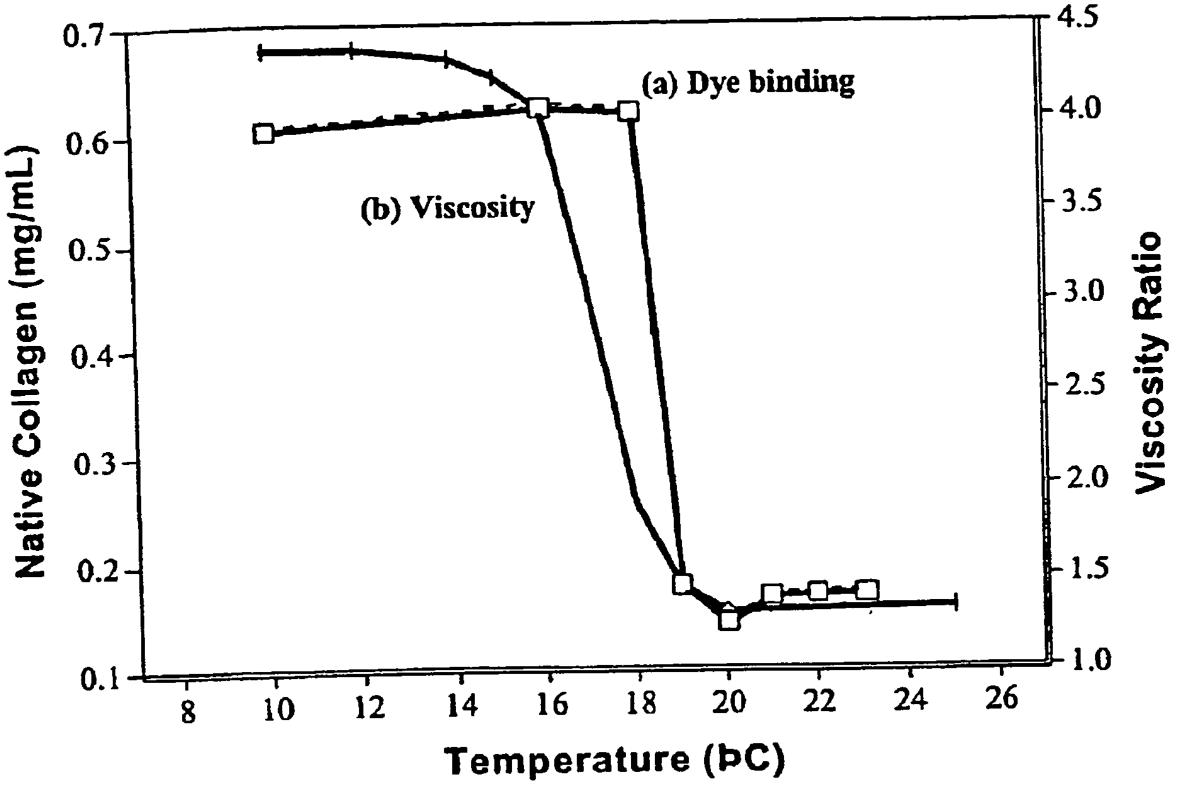


FIGURE 12



0.7
0.6
0.5
0.4
0.3
0.2
0.1
Native Collagen (mg/mL)
4.5
4.0
3.5
3.0
2.5
2.0
1.5
1.0
Viscosity Ratio
(a) Dye binding
(b) Viscosity
8
10
12
14
16
18
20
22
24
26
Temperature (þC)

COLLAGEN

[0001] This invention relates to collagen products and to methods for producing such products. In particular, it relates to collagen products extracted from the skin of cold water fish, suitable for use inter alia as a fining agent in the brewing and wine industries. Also provided is an assay for determining the amount of native collagen present in a protein sample.

BACKGROUND

[0002] Collagen products have a number of applications in various industries. In one such application, collagen finings are used in clarification or precipitation processes, for example for clarifying potable liquors such as beer and wine. During the fermentation of liquors various particulate materials such as yeasts and proteins become suspended in the liquor and need to be removed. Collagen finings are added to the liquor to clarify it by aiding the precipitation of the suspended materials.

[0003] Collagen finings are generally prepared from fish isinglass, which constitutes a very pure source of collagen prepared from the dried swim bladders of fish. The properties of such finings reflect their swim bladder derivation and the fact that the swim bladders are extracted from warm water fish such as members of the polynemidae family[2,3,4].

[0004] Another source of collagen is the skin. Many investigations have been made into the extraction of collagen from animal and fish skins including cold water fish skins (U.S. Pat. Nos. 4,295,894, 5,698,228, 5,162,506, 5,420,248, JP 4037679, JP 9-278639, JP 2-291814, PL 312122, RU 2139937). The collagen extraction processes known involve a wide range of chemical and mechanical extractions, or combinations thereof. The properties of the collagen products obtained by these processes vary widely. Many of the extraction processes applied to fish skins are adaptions of mammalian collagen extraction techniques. The applicants have identified that many of the processing steps applied to mammalian collagen extraction are not necessary for fish skin collagen extraction. Such steps including chemical washes and extractions, filtering, vacuum filtration, and decantation steps, and enzyme extractions amongst others. A simplified extraction process which eliminates many of these steps would be desirable.

[0005] It has also generally been accepted that collagen produced from skins of cold and deep water fish exhibits variable quality and is generally inferior to collagen produced from isinglass in finings production. It has therefore been concluded that cold and deep water fish skin collagen is unsuitable as a substitute for isinglass.[1]

[0006] The applicants have now surprisingly determined that it is possible to extract collagen from fish skin, specifically the skin of cold water fish, which collagen when extracted is suitable for use as a fining agent. Furthermore, the applicant's collagen has different and advantageous properties as compared to collagen finings from isinglass.

[0007] The present invention therefore provides collagen products which are, inter alia, suitable for use as finings.

[0008] It is therefore an object of this invention to provide a collagen product suitable for use as a fining agent, or at least to provide the public with a useful choice.

[0009] It is a further object of this invention to provide a simplified or alternate method for collagen extraction from cold water fish skins.

[0010] Collagen is recognised as a difficult and expensive protein to quantify because of the insoluble nature of most collagens. Yet, solubility is a key functional property important in a variety of applications such as healthcare products[5]. It has been determined that the conformation of the native collagen molecule determines molecular functionality, with transition to the random coiled confirmation of gelatin upon denaturing resulting in a significant loss in fining ability.

[0011] A number of methods are known for determining collagen concentration. These include determining hydroxyproline (unique to this protein) content[6], sophisticated gel electrophoresis and/or HPLC (requiring sample solubilisation) tests, kjeldahl for nitrogen, and polarimetry methods[7]. These methods are all complex and time consuming and require sophisticated and/or expensive equipment. It would therefore be a significant advantage to have a simple method for determining the amount of native collagen in a protein sample.

[0012] The applicants have unexpectedly found that total content of collagen in a sample from skins of cold water fish can be measured using the biuret method (Gornall et al.)[9] and that native collagen content can be quantified using dyes which bind only to native collagen. These assays taken together provide a simple, rapid method for determining native collagen concentration in a sample.

[0013] Accordingly, it is a further aspect of this invention to provide a method for determining native collagen concentration in a sample, or again at least to provide the public with a useful choice.

SUMMARY OF THE INVENTION

[0014] In one embodiment, the invention provides a fish skin collagen product in which native collagen forms 75% or more of the total protein content as measured by the Hofman test, which has an imino acid content of less than 170 per 1000 amino acid residues, and which meets, in addition, at least one of the following requirements:

[0015] (a) in solutions of about 6 mg/mL total solids dissolved in water, more than 60% is protein recoverable from the supernatant after centrifuging at 40,000× g for 30 minutes at 5° C.; and

[0016] (b) the intrinsic viscosity will be between 13 and 30 dL/g determined in 0.1 M acetic acid at 10° C.

[0017] By "native collagen", it is meant the tripeptide tropocollagen, consisting of three α-chains, substantially in its native soluble form.

[0018] Conveniently, more than 60% of the protein remains in the supernatant after centrifuging a range of solutions of between 2-10 mg/mL total solids dissolved in water at 40,000× g for 30 minutes at 5° C.

[0019] In a further embodiment, the invention provides a collagen product obtainable by stabilising a collagen paste by the addition of a preservative, said paste being the result of the following pre-stabilisation steps:

[0020] (i) without a prior extraction, effecting swelling of skins from cold water fish from which all residual flesh, fats and pigments have been removed, said swelling involving contacting said skins for an appropriate length of time with an acid solution; and

[0021] (ii) without drying said swollen skins, homogenising said skins to form a paste

[0022] each of which steps is conducted at a temperature below the thermal melting temperature (Tm) for the cold water fish species from which the skins are obtained.

[0023] As used herein, "cold water fish" means a fish having a skin containing protein in which the imino acid content is less than 170 per 1000 amino acid residues and/or the Tm is lower than 20° C. Examples of such fish include New Zealand hoki (also called blue grenadier), Atlantic cod, Alaska pollock, arrowtooth flounder, Pacific whiting, South African hake, South American hake, South American hoki, lingcod, and salmon.

[0024] Preferably, said skins in step (i) are undried.

[0025] Preferably, all residual flesh, fat and pigments are removed from said fish skin by a mechanical cleaning process such as an abrading or scraping process. It is particularly preferred that all residual flesh, fat and pigments be removed using tumble abrasion in water, or with water jets.

[0026] Conveniently, said skins are contacted with said acid solution of less than 0.5 M concentration for at least 30 minutes.

[0027] It is preferred that the acid be selected from acetic acid, citric acid and tartaric acid although inorganic acids may also be used.

[0028] Preferably, said skins are contacted with said acid solution at a ratio of from 10-200 g of skin per liter of acid.

[0029] Preferably, in said process the homogenised skins, in the form of a paste, are allowed to stand for at least 30 min at a temperature of less than 15° C., and desirably less than 12° C. prior to stabilisation.

[0030] Preferably, said preservative is a food growth preservative such as a sulphite, an antimicrobial organic acid or a salt thereof (eg. sorbate, acetic and lactic acids).

[0031] Said stabilised paste is either stored at a temperature of 12° C. or less or is dried.

[0032] Generally described is a process for the extraction of protein from fish skin wherein said protein is predominantly native collagen comprising providing a starting material consisting essentially of skin from cold water fish from which all flesh, fat and pigments have been removed, extracting protein from said fish skin and recovering said protein.

[0033] Preferably, said skins are undried.

[0034] In still a further embodiment, the invention provides a method for preparing a stabilised collagen paste which comprises the step of adding a preservative to a collagen paste, said paste having been obtained by the following pre-stabilisation step

[0035] (i) without a prior extraction, effecting swelling of skins from cold water fish from which all residual flesh, fats and pigments have been removed, said swelling involving contacting said skin for an appropriate length of time with an acid solution; and

[0036] (ii) without drying said swollen skins, homogenising said skins to form a paste,

[0037] each of which steps is conducted at a temperature below the Tm of the cold water fish from which the skins are obtained.

[0038] The invention also provides a method for preparing a pre-stabilised collagen paste which comprises the steps of:

[0039] (i) without a prior extraction, effecting swelling of skins from cold water fish from which all residual flesh, fats and pigments have been removed, said swelling involving contacting said skin for an appropriate length of time with an acid solution; and

[0040] (ii) without drying said swollen skins, homogenising said skins to form a paste,

[0041] each of which steps is conducted at a temperature below the Tm of the cold water fish from which the skins are obtained.

[0042] Preferably, said skins are undried.

[0043] It is also preferred that swelling is effected using an organic acid, for at least 30 minutes.

[0044] In still a further embodiment, the present invention provides a method for preparing a collagen product in which at least 75% of the total protein is in the form of native collagen, said process comprising the steps of

[0045] (i) removing all residual flesh, fat and pigments from fish skins by a mechanical cleaning process;

[0046] (ii) without a prior extraction, effecting swelling of said skins through contacting said skins with an acid solution for an appropriate length of time;

[0047] (iii) homogenising said swollen skins to form a paste;

[0048] (iv) holding the resulting paste for an appropriate period of time to allow the collagen present to complete swelling and solubilisation; and optionally

[0049] (v) adding a preservative to stabilise said paste.

[0050] In an alternate process step, (iii) may be effected prior to step (ii).

[0051] Preferably, in step (iv), said paste is held for a minimum of 30 minutes.

[0052] Preferably, said method further includes the step of:

[0053] (vi) drying the stabilised paste at a temperature below the Tm of the fish from which the skins were obtained.

[0054] In yet a further embodiment, the present invention provides a collagen product which is prepared by a process as defined above.

[0055] In one embodiment, the collagen product is in the form of a stabilised paste.

[0056] In an alternative embodiment, said collagen product is a dried product.

[0057] In still a further embodiment, the invention provides a composition which includes a collagen product as defined above.

[0058] In one embodiment, said composition is a foodstuff.

[0059] In another embodiment said composition is a fining agent.

[0060] In an alternative embodiment, said composition is a medicament, nutriceutical or cosmetic.

[0061] In still a further aspect, the invention provides a method of clarifying potable liquor to remove particulate matter present in said liquor which comprises the step of adding a collagen product as defined above to said liquor.

[0062] In a yet further aspect, the invention provides a method for determining the native soluble collagen content of a protein solution, the method comprising:

[0063] (a) determining the total protein content of the solution using a biuret assay;

[0064] (b) determining the amount of native collagen in the solution using a Sirius red dye binding assay; and

[0065] (c) comparing the results of (a) and (b) to give the proportion of native collagen in the protein solution; and

[0066] wherein step (b) is carried out at temperature less than the thermal melting temperature of the collagen being tested for.

[0067] In a still further aspect, the invention provides a kit for determining the amount of native collagen in a sample, the kit comprising copper sulphate, potassium sodium tartrate, and Sirius red.

DESCRIPTION OF THE DRAWINGS

[0068] While the present invention is broadly as defined above, it will be appreciated by those persons skilled in the art that it is not limited thereto but that it also includes embodiments of which the following description provides examples. In addition, a better understanding of the invention will be gained through reference to the accompanying drawings in which:

[0069] **FIG. 1** is a graph showing reduced viscosity versus concentration of freeze-dried hoki skin collagen in 0.1 M acetic acid determined at 10° C. The intercept of the vertical axis is the intrinsic viscosity for this hoki skin collagen, a value of 20.4 dL/g.

[0070] **FIG. 2** is a plot of viscosity ratio versus temperature for a 1 mg/mL hoki skin collagen solution in 0.5 M acetic acid. Solutions were held at the indicated temperature for 30 min prior to measurement in an Ubbelohde-type capillary viscometer (model #1, Cannon Instrument Co, PA, USA).

[0071] **FIG. 3** shows the solubility of hoki skin collagen in water, 0.1M acetic acid and 0.1M citric acid after solubilisation of the dry material (Before) and after centrifigation at 40,000× g for 30 min at 5° C. (After Centrifugation).

[0072] **FIG. 4** shows the fining effectiveness for chardonnay wine shown by a decrease in absorbance at 280 nm with increasing concentration of soluble hoki skin collagen. (a) Three hoki collagen preparations are shown freeze-dried material solubilised in 0.1 M citric acid and paste solubilised in both 0.1 M citric and 0.1 M acetic acid. (b) Freeze-dried hoki skin collagen solubilised in chilled 0.1 M citric acid at a concentration of 10 mg/mL prior to final dilution in the chilled wine.

[0073] **FIG. 5** shows L to R control (isinglass 25 mg/L), hoki skin collagen (10 mg/L) and untreated beer.

[0074] **FIG. 6** shows L to R Control (isinglass 20 mg/L), untreated wort, hoki skin collagen (20 mg/L).

[0075] **FIG. 7** shows fining ability of hoki skin collagen compared to a commercial isinglass product (Control). Hoki skin collagen was added to unfined beer at 12 mg/L and the Control was added at 22 mg/L.

[0076] **FIG. 8** shows comparison of clarity and stability of beer fined with either hoki skin collagen or a commercial isinglass preparation (Control). Haze at 90° and 25° forward scatter (EBC units) after 1 month and 2 month's storage. Hoki skin collagen was added to unfined beer at 12 mg/L and the Control was added at 22 mg/L.

[0077] **FIG. 9** shows sensory analysis of chardonnay wine from fining trials using four fining agents including hoki skin collagen. Shows increased fruitiness and reduced astringency when hoki skin collagen was used as a fining agent (0.03 g/L concentration hoki skin collagen and stored for 96 hr at 10° C. prior to analysis).

[0078] **FIG. 10**. Standard curve for biuret method of protein estimation using hoki skin collagen disolved in 0.1 M acetic acid.

[0079] **FIG. 11**. (*a*) The precipitation of the dye Sirius Red by hoki skin collagen at 10° C. (□), as shown by a decrease in absorbance at 540 nm. (*b*) Loss of ability to quantitatively precipitate the dye after hoki collagen was heated to 30° C. for 10 min prior to assay (◇).

[0080] **FIG. 12**. (*a*) Concentration of hoki collagen in solution with change in temperature (□) as measured by the binding of Sirius Red F3BA dye. Each sample point is the average of five dye assays. (*b*) The change in viscosity ratio with temperature (◇) for a 1 mg/ml hoki collagen solution in 0.5M acetic acid. Each sample point is the average of three viscosity measurements.

DESCRIPTION OF THE INVENTION

[0081] As broadly defined above, the primary focus on the present invention is on a collagen product. This collagen product has application in the liquor, food, cosmetic and healthcare industries.

[0082] The essential finding of the applicants is that it is possible to extract collagen from the skins of cold water fish in a manner such that the resulting extracted collagen has advantageous properties. Those properties make the resulting collagen particularly suitable for a number of applications, including use in the clarification of potable liquors such as beer and wine.

[0083] Examples of cold water fish from which skin can be obtained for use as the starting material for the extraction process of the invention include New Zealand hoki (also called blue grenadier), Atlantic cod, Alaska pollock, arrowtooth flounder, Pacific whiting, South African hake, South American hake, South American hoki, lingcod, and salmon. However, this list is by no means exhaustive and should not be interpreted as limiting.

[0084] One primary quality of the collagen of the invention is that at least 75% of the total protein present is native collagen. The percentage of native collagen present is measured by the Hofman test.

[0085] By the "Hofman test" it is meant a novel combination of two assays for determining the relative proportion of a protein solution that is made up of native collagen. Specifically, total protein in the solution is determined by the biuret reaction which uses a copper complex to measure the number of peptide bonds, whereas native collagen is determined by the ability of the dye Sirius Red to quantitatively bind to native collagen molecules and cause the collagen molecules to precipitate from solution.

[0086] The applicants have determined that the biuret reaction will give a linear standard curve up to a concentration of 6 mg/mL using freeze-dried fish skin collagen. Briefly, 0.5 mL of sample (1-5 mg/mL protein) is added to 2.5 mL biuret reagent (0.006 M copper sulphate, 0.02 M potassium sodium tatrate in strong NaOH solution) mixed and the absorbance at 540 nm is read after 20 min. The absorbance is calibrated against a standard curve (0-5 mg/mL protein) using hoki skin collagen to give the total protein present.

[0087] The applicants have further determined that the dye binding assay using Sirius Red binds only to native collagen molecules, and can be used in the range 5 to 65 μg collagen. To 5-65 μg protein in 100 μL 0.5 M acetic acid is added 1.0 mL Sirius Red solution (50 μM Sirius Red in 0.5 M acetic acid). After 30 min samples are centrifuged at 11,000× g and the absorbance of the supernatant is read at 540 nm. The absorbance is calibrated against a standard curve using samples of 5-65 μg collagen in 0.5 M acetic acid.

[0088] Comparison of the results from the biuret and the dye binding assays gives the relative proportion of native collagen in the protein solution.

[0089] Dye binding is carried out at a temperature less than the thermal melting temperature (Tm) for that collagen. The Tm is a measure of the transition from soluble collagen molecules to denatured collagen chains or gelatin and is defined as the temperature at which half of the initial intrinsic viscosity disappears in 30 minutes.

[0090] Accordingly, the present applicants have been the first to provide teaching of a rapid, simple and reliable test for determining the proportion of native collagen in a protein solution, especially collagen from cold water fish skins. The Hofman test therefore forms a further aspect of this invention.

[0091] The Hofman test also forms the basis of a kit for the determination of native collagen in a protein sample. Accordingly, also provided by the invention is a kit suitable for use in the Hofman test. The kit comprises the assay reagents, namely copper sulphate, potassium sodium tartarate, and Sirius Red. The reagents may be made up in solution ready for use. The reagents are separately contained, and are provided in amounts sufficient for carrying out at least one, and preferably multiple Hofman tests. The kit may further comprise a standard for the collagen being tested.

[0092] In addition, the collagen product of the invention is defined by reference to functional criteria. These include solubility in water and intrinsic viscosity.

[0093] In terms of solubility, in solutions of between 2-10 mg/mL total solids dissolved in water, more than 60% is protein recoverable from the supernatant after, centrifuging at 40,000× g for 30 minutes at 5° C. Conveniently, the solubility measurement is made at a concentration of between 4-8 mg/mL and most usually at about 6 mg/mL in water, and protein recovery is more then 70%.

[0094] As for intrinsic viscosity, this will be between 13 and 30 dL/g determined in 0.1 M acetic acid at 10° C. using an Ubbelohde-type capillary viscometer (model #1, Cannon Instrument Co, PA, USA).

[0095] The invention also provides a collagen product obtainable by stabilising a collagen paste by the addition of preservative. The paste is pre-stabilised by swelling defleshed, defatted, depigmented cold water fish skins in acid solution. The defatting, defleshing and depigmentation is effected by mechanical cleaning processes known

[0096] in the art, including abrading and scraping. Preferably, flesh, fat and pigments are removed by tumble abrasion in water or using water jets.

[0097] Acid solutions may be inorganic acids such as hydrochloric or sulphuric acid, but are preferably organic acids such as acetic, citric and tartaric acid but are not limited thereto. Dilute acid solutions of from 0.05 to 0.8M are generally used. Preferably, 0.1 to 0.5M.

[0098] The skins are contacted with the acid solution at a ratio of from 10-200 g of skin per liter of acid. The ratio is dependent on the type of collagen product desired. More concentrated pastes (5-50 mg collagen/mL) are suitable for shipping and storage while dilute solutions (8 mg collagen/mL and lower) may be more convenient for actual use.

[0099] Swelling time is desirably at least 30 minutes, up to 1 or 2 days, or even weeks if the paste is to be stored prior to stabilisation.

[0100] In a preferred process the homogenised skins, in the form of a paste, are allowed to stand for at least 30 minutes, or for hours, days or weeks at a temperature less than 15° C., and most desirably less than 12° C.

[0101] Homogenisation is carried out using standard art techniques. All steps are conducted at a temperature below the thermal melting temperature for the cold water fish species from which the skins are obtained. This avoids denaturation and loss in functionality of the collagen product.

[0102] The preservative added may be any preservative suitable for use in foodstuffs. This includes sulphite, antimicrobial organic acids or salts thereof but are not limited thereto. Preferred preservatives are sulphite, sorbate, acetic acid and lactic acid.

[0103] The skins can be fresh, frozen or dried. In a currently preferred embodiment the skins are undried.

[0104] The solubilised paste is stored in the same conditions as the paste pre-stabilisation, or is dried. Drying may be carried out using known art techniques. Preferably freeze drying is carried out keeping heating temperatures below the Tm of the collagen.

[0105] In a related aspect, the invention also provides a method for preparing a stabilised collagen paste by adding a preservative to a collagen paste pre-stabilised according to the above methods.

[0106] This invention also provides a process for extracting protein from fish skin, which protein is predominantly native collagen. As discussed above, the starting material consists essentially of skin from cold water fish. This skin is subjected to flesh, fat and pigment removal as discussed. Protein extraction is then effected by swelling the skins with a dilute acid solution for an appropriate length of time.

[0107] Any of the acids referred to above may be used. Preferably, the organic acids are used. The extraction step is carried out for at least 30 minutes and usually for hours, or even several days. The protein solubilised in the acid is then recovered as a paste.

[0108] Again, the skins used in the process can be fresh, frozen or dried. In a currently preferred embodiment the skins are undried.

[0109] The collagen product of the invention is obtainable from cold water fish skins essentially in accordance with the following (preferred) process steps.

[0110] 1. Raw material

[0111] Skins are obtained from the skinning operation from either fresh or frozen fish, alternatively frozen skins are thawed.

[0112] 2. Cleaning

[0113] The skins are subjected to mechanical cleaning by abrasion or scraping in tap water. Residual flesh, fats and pigments are removed by scraping, in tumble abrasion or using water jets. The process is repeated until the skins are sufficiently clean. These skins may be used directly or frozen or dried for later manufacture.

[0114] 3. Swelling

[0115] Clean skins are swollen for 30 minutes or more in dilute organic acid (acetic, citric, tartaric or other) at a ratio of 10-200 g per liter clean skin to acid.

[0116] 4. Communition

[0117] Swollen skins are homogenised in a vertical cutter/mixer or similar machine to make a fine paste.

[0118] 5. Extraction

[0119] The fine paste is held for a period of time to allow the collagen fibers to complete swelling and solubilising.

[0120] 6. Storage

[0121] The collagen paste is stabilised with acceptable preservatives and stored at temperatures below 12° C. Preservatives that may be used are sulphite, antimicrobial organic acids or their salts (eg. sorbate, acetic and lactic acids) and other preservatives commonly used in the food industry.

[0122] The collagen product may be retained in the form of the stabilised paste or can be part of a solution. It is also possible to provide the collagen product in a dry form, in which case it is preferred that the process include the following step:

[0123] 7. Freeze drying

[0124] Solubilised collagen is dried at temperatures less than the Tm of the fish species from which the skins were derived for sale as a dry product.

[0125] In step (1) the preferred mechanical cleaning process is water abrasion. Acid swelling of skins may also be effected using any of the acid solutions discussed above, and for the times given. In step (3) the paste is usually held for a minimum of 30 minutes and more usually hours or days.

[0126] The order of steps 3 and 4 may also be reversed. That is, communition may be effected prior to swelling.

[0127] A wide range of communition techniques and apparatus known in the art may be used as alternatives to the vertical cutter/mixers.

[0128] Extraction step 5 as noted above is carried out for at least 30 minutes, and preferably for hours or days to allow the collagen fibres to complete swelling and solubilising.

[0129] The optional freeze drying step is also effected using standard art techniques, desirably keeping heating temperatures below the Tm of the collagen.

[0130] The invention also relates to collagen products produced by the processes of the invention. The collagen products may be used as prepared, or may be incorporated in compositions including foodstuffs, such as hydrocolloids, or in medicaments, nutraceuticals or cosmetics. These applications for collagen are well documented in the art.

[0131] In a further aspect the invention also provides a method of clarifying potable liquor such as beer or wine to remove particulate matter. As with the use of known isinglass collagen products, collagen is mixed in with the liquor and allowed to settle. This removes yeast cells and/or other suspended components of the liquor. The collagen product or paste of the invention may be used. Preferably, the product is the paste in acid, or freeze dried collagen in acid. The collagen is added in an amount of from 0.005 to 0.5 g/L, preferably 0.01 to 0.4 g/L, and more preferably 0.02 to 0.1 g/L. For beer the collagen is most usually added in an amount of from 0.02 to 0.1 g/L. For wine the collagen is most usually added in an amount of from 0.01 to 0.04 g/L.

[0132] The collagen product of the invention is highly effective in clarifying both beer and wines. Stored beer products showed showed much lower haze levels than beer treated with current isinglass fining products. Compared to known isinglass products the collagen products of the present invention worked at least twice as fast, and removed more suspended product in beer and wort. In red wines colour was better using this product, while red and white wines showed softening of flavour, namely reduced astringency and unmasking of fruit flavour, retention of tannins for flavour structure, as well as good retention of volatile flavour components, and less lees production. Hoki skin collagen is therefore a useful fining agent.

[0133] The invention will now be illustrated with reference to the following non-limiting examples.

EXAMPLE 1

[0134] Extraction of Collagen Product from Hoki Skin.

[0135] Fresh hoki were skinned using a Trio skinning machine (Trio, model FDS-2N skinner, Sweden). The skins were then added to a vertical cutter/mixer (Stephan Machinery, NZ) with a ratio of 1.5 kg skins to 1000 mL ice/water slurry. The mixture was comminuted for 1 min on speed setting one after which the skins were separated from the waste water by sieving. The skins were added back to the vertical cutter/mixer with 1000-2000 mL ice/water slurry and the tumble abrasion process was repeated for a total of 10 times. The cleaned skin tissue (110 g) was then added to 3000 mL of chilled O. 1M acetic acid and allowed to swell for 90 min. The mixture was then comminuted in the vertical cutter/mixer for 2 min on setting 1 to make a fine viscous paste of about 8 mg/mL collagen. The material was kept below 12° C. during all processing steps.

[0136] The resulting paste can be stored for up to 12 months under chill conditions (4° C.) prior to use or used directly in applications such as fining. For fining the paste is typically diluted prior to use to give a final concentration between 1 and 80 ppm depending on the final product attributes required.

[0137] Alternatively, the paste was freeze-dried to produce a dry, shelf stable material with a moisture content less than 12%. In this case paste was spread thinly on dryer trays, frozen to below -20° C. and freeze-dried for up to 48 hr. Low temperatures during drying were maintained by circulating tap water (18-20° C.) to heat the dryer trays. Typically the product temperature was maintained below 0° C. for the constant drying rate period which finished about 12 hr from the start of drying. Following completion of drying product was removed from the trays by scraping. The dry product was then packed into heat sealable aluminium laminate bags for long term storage. A yield of 1.5 g dry protein from 10 g wet cleaned skins can be attained with the resultant product having greater than 75% native collagen as determined by the Hofman test.

[0138] **FIG. 1** shows results to determine the intrinsic viscosity of freeze-dried extracted hoki skin collagen. For this material the intrinsic viscosity in 0.1 M acetic acid was 20.4 dL/g as shown by the intercept of the reduced viscosity versus concentration plot (**FIG. 1**).

[0139] **FIG. 2** shows results for determining the Tm, thermal melting temperature, of 17.2° C. for hoki skin collagen. The Tm is a measure of the transition from soluble collagen molecules to denatured collagen chains or gelatin and is defined as the temperature at which half of the initial intrinsic viscosity disappears in 30 minutes.

[0140] **FIG. 3** shows the results of solubility studies demonstrating the highly soluble nature of hoki skin collagen in water and in dilute organic acid solutions. For example, after centrifugation to remove colloidal particles, and over the concentration range 2-10 mg/mL added protein, more than 70% of the protein remained dissolved in water. Similar results were gained when 0.1M acetic or citric acid were the solvent.

[0141] Briefly, freeze-dried protein extracted from hoki skin (as described herein) was mixed with 100 mL solution and stirred for 60 min (8° C.). The mixture was then homogenised with an Ultraturex mixing tool (IKA-Laboratechnik, model T25, Germany) using two 10 see bursts at 13,500 rpm. The solutions were shaken for a further 48 hr (8° C.), after which the total protein concentration was determined by biuret. The solutions were centrifuged for 30 min at 40,000× g (5° C.) and the protein content of the supernatant was measured by biuret to determine the amount of protein remaining in solution.

EXAMPLE 2

[0142] Use of Hoki Skin Collagen as a Fining Agent in Wine

[0143] A stock solution of 5 mg/mL hoki skin collagen in chilled 0.1 M citric or acetic acid was made by stirring until the paste or freeze-dried material was dissolved. Aliquots of the stock solution were then added to 50 mL prechilled chardonnay wine in a 50 mL measuring cylinder to give a final concentration between 0 (control) and 60 ppm. Sufficient chilled water was also added to give a constant dilution for all treatments. The measuring cylinder was covered with Parafilm and gently inverted to mix the fining agent and wine and then stored at 8° C. for 24 hr. The clarified wine was then filtered through no. 1 Whatman filter paper, diluted 1:10 with distilled water and the absorbance at 280 nm was determined in a spectrophotometer (Univam, model UV4-100) using water as a blank.

[0144] FIGS. 4 ((*a*) and (*b*)) demonstrates the effectiveness with increasing concentration of two forms of hoki skin collagen (paste dissolved in acetic and citric acid; and freeze-dried collagen in citric acid) for removal of phenolics and proteins associated with turbidity and bitter or astringent flavours in white wine.

EXAMPLE 3

[0145] Use of Hoki Skin Collagen as a Fining Agent in Brewing

[0146] Introduction

[0147] Laboratory scale trials and an initial trial in 50 L kegs were carried out using a collagen product of the invention with potential use in the fining of beer and wine. The product was presented in a solid form and as a paste. These trials showed good fining properties. The trials were repeated on a 50 L basis to judge the effect on other beer parameters such as taste, head retention and haze stability.

[0148] Summary

[0149] Trial products were added to unfined beer at 12 mg/L and performance compared to a control of current commercial products sourced from Biocon (Quest International, NZ) and AB Vickers Ltd (UK) added at the current rate. The collagen product of the invention was found to be an effective fining agent.

[0150] Results

[0151] Results of Studies Using Hoki Skin Collagen in Brewing

[0152] After laboratory and industrial trials, hoki skin collagen has been found to be a highly effective fining agent for beer and wort. Hoki skin collagen is more effective than some commercially available isinglass-based preparations. In addition, hoki skin collagen confers excellent chill-proofing capability, haze stability on storage and has no effect on taste and head retention of beer.

[0153] Ability to Fine

[0154] **FIGS. 5 and 6** show the excellent fining ability of hoki skin collagen compared to the commercially available isinglass preparation (Control). In these small-scale trials hoki skin collagen settled within 24 hr while the Control sample of beer and wort took approximately twice as long to clear. For beer the dosage rate for hoki skin collagen was less than half that needed for the Control.

[0155] **FIG. 7** shows the results from larger scale trials in which hoki skin collagen treated beer cleared faster and removed much more suspended yeast than the commercial isinglass-based product.

[0156] Clarity and Stability

[0157] **FIG. 8** shows results of a storage trial in which hoki skin collagen treated beer was clearer and more stable during storage compared to the Control. After 2 months storage hoki skin collagen treated beer had a haze reading (90°) about 70% lower than for the Control treated beer.

EXAMPLE 4

[0158] Use of Hoki Skin Collagen as a Fining Agent in White and Red Wine

[0159] In white wines isinglass has been linked to a reduction in browning potential. It achieves the above without unduly decreasing the tannin concentration, or affecting the colour of the wine. The result is a brilliant, clear and softer wine. Isinglass is typically added at a rate of 0.02 to 0.1 g/L (20-100 ppm) producing compact lees of approx. 2%.

[0160] Herein we show experimental results for use of hoki skin collagen as a fining agent in white and red wine. Hoki skin collagen performs similarly to commercially available isinglass-based preparations. However, because of its improved functional properties hoki skin collagen has additional benefits associated with it including less loss of colour in light red wines such as Pinot noir, softening of flavour in white and red wines, and retention of tannins for flavour structure.

[0161] Collagens from skin of cold-water fish species provide further advantages for fining of wines made at low temperatures to retain volatile flavour components associated with aromatic wines such as sauvignon blanc, reisling and gwertztraminer.

[0162] Experimental

[0163] Pinot noir wine was from a barrel sample. A stock solution of 0.6% w/v was made up of freeze-dried hoki skin collagen by adding 0.2 g dry material to 33 mL deionised water. This was stirred for 2 min, left for 15 min to swell, and then stirred again to give a clear solution with no visible clumps. Treatments were performed in duplicate. Stock solution was slowly dispensed into 100 mL wine in a 100 mL measuring cylinder that had been sparged with CO_2 to give the following final concentrations; 0, 0.01, 0.02, 0.03, 0.04 and 0.1 g/L. The 0.1 g/L concentration was used to give an indication of the consequences of overfining with the agent. The treatments were then covered with Parafilm and mixed by gentle inversion 5 times, then left undisturbed in a 15° C. temperature controlled room for 48 hr.

[0164] The treatments were then carefully decanted off their lees into two 50 mL centrifuge tubes and spun at 4500 rpm, at −10° C. for 10 min. Samples were then vacuum filtered through 0.45 μm filters (Millipore Corp., USA). Sets of 10 mL samples were retained for specrophotometric analysis.

[0165] Spectrophotometric Analysis

[0166] Spectrophotometric analysis of wine colour parameters ($A_{420\ nm}$; a measure of "brown" hue, $A_{520\ nm}$; a measure of "red" hue, colour density; $A_{420\ nm}+A_{520\ nm}$, and colour hue; $A_{420\ nm}/A_{520\ nm}$, and of total phenolics; $A_{280\ nm}-4$. All analyses were performed on a Unicam UV4-100 spectrophotometer controlled by Vision v3.10 software (Unicam, UK).

[0167] Results

[0168] Hoki skin collagen produced a light "fluffy" lees layer that was easily disturbed but compact after 36 hr at 15° C. This layer was more compact than those produced by commercial isinglass preparations (refer **FIG. 9**) under the same conditions. The actual depth of the lees layer was related to the amount of agent added with about 7% percent lees produced when 0.04 g/L hoki skin collagen was added. Observation of the wine post settling indicated the fined wine had an increased clarity ("brightness").

[0169] Effect of Fining with Hoki Skin Collagen on Wine Colour Parameters and Total Phenolics:

[0170] Table 1 shows that treatment with hoki skin collagen did not significantly alter wine colour parameters and total phenolics when the material was used within typical/practical limits. That is, when an application range between 0.01 and 0.04 g/L was used for clarification. The greater reduction in absorbance at 420 nm and 520 nm with the addition of 0.1 g/L shows that hoki skin collagen can alter phenolic composition and colour of the wine at higher use levels. This may be of benefit when a wine maker may wish to reduce "browning" in a wine, even at the expense of a reduction in some of the "red" hue. Similarly, higher concentrations of hoki skin collagen may be used to remove phenolics contributing to undesirable levels of bitterness and astringency. However, colour hue and total phenol content of the wine was not significantly altered by any of the addition rates of hoki skin collagen tested. hoki skin collagen is a useful fining agent, particularly with Pinot noir wine.

TABLE 1

Effect of hoki skin collagen treatments on Pinot noir wine

Protein Conc (g/L)	A_{420nm}	A_{520nm}	Colour Density	Colour Hue	Total Phenolics
0	1.94	2.28	4.22	0.854	4.29
0.01	1.92	2.24	4.17	0.857	3.65
0.02	1.91	2.22	4.13	0.863	3.98
0.03	1.92	2.24	4.17	0.857	4.63
0.04	1.92	2.23	4.15	0.859	3.91
0.1	1.80	2.09	3.89	0.858	3.67

EXAMPLE 5

[0171] Application of Hofman Test to Hoki Skin Collagen

[0172] Introduction

[0173] Solubility of collagen is a key functional property that is important in a variety of applications. Collagens extracted from cold-water fish species are not only more labile but also have interesting functional properties, such as high solubility in dilute acid and increased ability to interact with colloidal particles.

[0174] The performance of finings has been shown to be related to the amount and size of the collagen molecules in solution (Leach and Barrett 1967)[4]. The conformation of the native collagen molecule determines molecular functionality since the transition to the random coiled conformation of gelatin results in significant loss in fining ability. Therefore, it would be a significant advantage to have a simple testing method to not only measure the amount of soluble collagen in a solution but also to quantitate the amount of native collagen, the more functional state.

[0175] Further, it has been found that collagen solubility is an important functional property for use in a range of healthcare products. For example, the use of collagen sponges as a matrix for bone or cartilage remodelling, for slow release of drugs or as a wound dressing (Chvapil 1979)[5].

[0176] The insoluble nature of most collagens makes collagen a difficult protein to quantify. The methods that are used to determine collagen concentration are expensive and time consuming. These include, determining hydroxyproline content, kjeldahl for nitrogen, gel electrophoresis, chromatography and polarimetry methods. There is, therefore, a need for a simple, rapid method for determining total collagen and native collagen concentrations.

[0177] Experimental

[0178] Collagen Preparation:

[0179] Hoki skins were scraped clean of all adhering tissue and scales and washed in distilled water. The skin was then swollen in 0.1 M acetic acid in a ratio of 1:42 (g/mL), homogenised in a food processor (Magimix, Australia), and held for 18 hr at 8° C. The extracted material was then centrifuged for 60 min at 10,000× g (5° C.). The supernatant was diluted and taken to 1.0 M NaCl with the addition of 4.4 M NaCl solution. Following centrifugation for 60 min at 8,000× g (5° C.) the pellet was dissolved in 0.1M acetic acid and dialysed extensively before freeze drying for storage.

[0180] Total Protein Assay:

[0181] Total protein concentrations were measured according to the biuret method (Gornall et al., 1949)[9] using hoki skin collagen as standard. Briefly, 1.5 g copper sulphate and 6.0 g sodium potassium tartrate was dissolved in 500 mL water. Then 300 mL of 10% sodium hydroxide was added and the solution was made up to 1 L with water to make the biuret reagent. To 2.5 mL biuret reagent was added 0.5 mL protein sample, the mixture was vortexed and held for 20 min at room temperature before determining the absorbance at 540 nm.

[0182] Native Collagen Assay:

[0183] The ability of the dye Sirius Red F3BA to quantitatively precipitate collagen in acid solution was used to determine the concentration of native collagen in a solution (Marotta and Martino 1985)[8]. Native collagen was assayed by adding 100 μL protein solution (5-65 μg protein in 0.5 M acetic acid) to 1.0 mL Sirius Red F3BA dye (50 μM in 0.5 M acetic acid). After 30 min the samples were centrifuged (11,600× g for 8 min) to remove precipitated collagen and the absorbance of the supernatant was determined at 540 nm.

[0184] Collagen Thermal Denaturation Studies:

[0185] Hoki skin collagen (0.7 mg/mL) was dissolved in 0.5 M acetic acid and samples were held for 30 min at temperatures from 10-23° C. After heating, samples were rapidly cooled in an ice slury and the concentration of the native collagen present was determined by the ability of the protein to bind Sirius Red F3BA dye as described above.

[0186] To compare the dye binding assay to another standard measurement we measured the change in viscosity as hoki skin collagen (1 mg/mL in 0.5 M acetic acid) was heated for 30 min at temperatures from 10-25° C. Viscosity was measured using an Ubbelohde-type capillary viscometer (model #1, Cannon Instrument Co, PA, USA).

[0187] Results

[0188] Biuret for Determining Total Protein

[0189] We used the biuret method for determining total collagen (or total protein) concentration of hoki skin collagen solutions over the range 0-6 mg/ml. **FIG. 10** shows that hoki skin collagen behaves linearly with the biuret method of protein determination for concentrations up to 6 mg/ml. The biuret method was not affected by pretreatment temperatures that denatured all hoki collagen present in solution.

[0190] Dye-Binding Method for Determining Native Collagen

[0191] **FIG. 11*a*** shows the precipitation of the dye Sirius Red by hoki skin collagen when collagen solutions are stored and assayed below the collagen Tm. **FIG. 11*b*** shows the loss of dye binding ability when the hoki collagen was heated at 30° C. for 10 min prior to assay.

[0192] **FIG. 12** shows the change in native collagen concentration as measured by dye binding (a) and viscosity (b) after heating at the indicated temperature for 30 min. The dye binding method followed changes in viscosity as the collagen molecules denatured and changed from a rod shape to a more globular structure. Dye binding was slightly less sensitive than the viscometric method to initial molecular shape changes in the initial part of the collagen to gelatin transition. This is demonstrated by an abrupt transition between 18 and 19° C. compared to the more gradual change in viscosity from 16 to 20° C. The transition temperatures derived from each method are close with 18.5° C. and 17.3° C. for dye and viscosity respectively.

[0193] Method for Determining Percentage of Total Protein That is Native Collagen

[0194] The dye Sirius Red F3BA only binds to native collagen molecules and will not bind to denatured collagen molecules. Therefore, a combination of the biuret and dye-binding methods gives specific information on the total quantity of collagen (or total protein) in solution and quantity that is in the native state. Conversely, the percent collagen that has been denatured can also be determined. This is valuable information for a number of industries that use collagen for its functional properties.

[0195] We have named this combined test the "Hofman Test"

[0196] In the fining industry the rapid and simple Hofman Test will make it possible to establish an industry standard test because of the difficulty of determining collagen content in products currently marketed and the lack of consistency with which this type of information is supplied by the producers of isinglass products.

INDUSTRIAL APPLICATION

[0197] Thus, in accordance with the present invention there is provided a collagen product which possesses a range of advantageous functional properties. These properties reflect the derivation of the collagen from the skin of cold water fish and also the process of extraction.

[0198] The resulting collagen product has application in numerous industries. These include the food industry, the healthcare industry and the cosmetic industry.

[0199] One specific application of the collagen product of the invention is in the clarifying of potable liquors, particularly beer and wine. The applicants have found that their collagen product is able to be used as a fining agent. Furthermore, the results achieved are equal to if not better than those achieved using the traditional isinglass fining agents.

[0200] The invention also provides a collagen extraction process adapted for use on cold water fish skins. This process eliminates many unnecessary processing steps previously considered essential or important for mammalian and/or fish skins. The process is therefore simple and easy to carry out.

[0201] The Hofman test and related kits also make it possible to monitor an extraction process, to develop effective purification procedures, to monitor samples during storage, and to develop a collagen product to a required specification.

[0202] It will be appreciated by those persons skilled in the art that the above description is provided by way of illustration only and that changes and modifications, particularly to the preparative process, may be made without departing from the scope of protection claimed.

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1. A fish skin collagen product in which native collagen forms 75% or more of the total protein content as measured by the Hofman test, which has an imino acid content less than 170 per 1000 amino acid residues, and which meets, in addition, at least one of the following requirements:

(a) in solutions of about 6 mg/mL total solids dissolved in water, more than 60% is protein recoverable from the supernatant after centrifuging at 40,000× g for 30 minutes at 5° C.; and

(b) the intristic viscosity will be between 13 and 30 dL/g determined in 0.1 M acetic acid at 10° C.

2. A collagen product as claimed in claim 1 wherein for (a) in solutions of between 2-10 mg/mL total solids dissolved in water, more than 60% is protein recoverable from the supernatant after centrifuging at 40,000× g for 30 minutes at 5° C.

3. A collagen product as claimed in claim 1 or claim 2 wherein for (a) more than 70% is protein recoverable from the supernatant after centrifuging.

4. A collagen product obtainable by stabilising a collagen paste by the addition of a preservative, said paste being the result of the following pre-stabilisation steps:

(i) without a prior extraction, effecting swelling of skins from cold water fish from which all residual flesh, fats and pigments have been removed, said swelling involving contacting said skins for an appropriate length of time with an acid solution; and

(ii) without drying said swollen skins, homogenising said skins to form a paste

each of which steps is conducted at a temperature below the thermal melting temperature (Tm) for the cold water fish species from which the skins are obtained.

5. A collagen product as claimed in claim 4 wherein the skins in step (i) are undried.

6. A collagen product as claimed in claim 4 or claim 5 wherein residual flesh, fats and pigments are removed by a mechanical cleaning process.

7. A collagen product as claimed in claim 6 wherein the mechanical cleaning process is tumble abrasion in water or uses water jetting.

8. A collagen product as claimed in any one of claims 4 to 7 wherein in step (i) the skins are contacted with said acid solution of less than 0.5 M concentration for at least 30 minutes.

9. A collagen product as claimed in any one of claims 4 to 8 wherein in step (i) the acid is selected from acetic acid, citric acid, and tartaric acid.

10. A collagen product as claimed in any one of claims 4 to 9 wherein in step (i) the skins are contacted with said acid solution at a ratio of from 10-200 g of skin per liter of acid.

11. A collagen product as claimed in any one of claims 4 to 10 wherein the homogenised skins in the form of a paste, are allowed to stand for at least 30 minutes at a temperature of less than 12° C. prior to stabilisation.

12. A collagen product as claimed in any one of claims 4 to 11 wherein said preservative is a food growth preservative.

13. A collagen product as claimed in claim 12 wherein the food growth preservative is selected from sulphite, an anti-microbial organic acid or salt thereof.

14. A collagen product as claimed in claim 13 wherein said organic acid is selected from sulphite, sorbate, acetic acid and lactic acid.

15. A collagen product as claimed in any one of claims 4 to 14 wherein said stabilised paste is dried.

16. A collagen product as claimed in any one of claims 4 to 14 wherein said paste is stored at a temperature of 15° C. or less.

17. A collagen product as claimed in claim 16 wherein said paste is stored at a temperature of 12° C. or less.

18. A method for preparing a pre-stabilised collagen paste which comprises the steps of:

(i) without a prior extraction, effecting swelling of skins from cold water fish from which all residual flesh, fats and pigments have been removed, said swelling involving contacting said skin for an appropriate length of time with an organic acid solution; and

[ii] without drying said swollen skins, homogenising said skins to form a paste,

each of which steps is conducted at a temperature below the thermal melting temperature of the cold water fish from which the skins are obtained.

19. A method for preparing a stabilised collagen paste which comprises the step of adding a preservative to a collagen paste, said paste having been obtained by the following pre-stabilisation steps:

(i) without a prior extraction, effecting swelling of skins from cold water fish from which all residual flesh, fats and pigments have been removed, said swelling involving contacting said skin for an appropriate length of time with an acid solution; and

(ii] without drying said swollen skins, homogenising said skins to form a paste,

each of which steps is conducted at a temperature below the Tm of the cold water fish from which the skins are obtained.

20. A method as claimed in claim 18 or claim 19 wherein the skins in step (i) are undried.

21. A method as claimed in claim 20 wherein the skins in step (i) are dried.

22. A method for preparing a collagen product in which at least 75% of the total protein is in the form of native collagen, said process comprising the steps of:

(i) removing all residual flesh, fat and pigments from fish skins by a mechanical cleaning process;

(ii) without a prior extraction, effecting welling of said skins through contacting said skins with an organic acid solution for an appropriate length of time;

(iii) homogenising said swollen skins to form a paste;

[iv] holding the resulting paste for an appropriate period of time to allow the collagen present to complete swelling and solubilisation.

23. A method as claimed in claim 22 wherein in step (iv), said paste is held for a minimum of 30 minutes.

24. A method as claimed in claim 21 or claim 22 wherein step (iii) is carried out prior to step (ii).

25. A method as claimed in any one of claims 22 to 24 wherein said method further includes the step of;

(v) adding a preservative to stabilise said paste.

26. A collagen product produced .by a process as claimed in any one of claims 18 to 25.

27. A collagen product as claimed in claim 26 wherein said product is in the form of a stabilised paste.

28. A collagen product as claimed in claim 26 wherein said product is a dried product.

29. A composition which includes a collagen product as claimed in any one of claims 1 to 17 and 26 to 28.

30. A composition as claimed in claim 29 wherein the composition is a foodstuff.

31. A composition as claimed in claim 29 wherein the composition is a fining agent.

32. A composition as claimed in claim 29 wherein the composition is a medicament, nutraceutical or cosmetic.

33. A method of clarifying potable liquor to remove particulate matter present in said liquor which method comprises the step of adding a collagen product as claimed in any one of claims 1 to 17 and 26 to 28, or a composition as claimed in claim 29 to said liquor.

34. A method for determining the native soluble collagen content of a protein solution, the method comprising:

(a) determining the total protein content of the solution using a biuret assay;

(b) determining the amount of native collagen in the solution using a Sirius red dye binding assay; and

(c) comparing the results of (a) and (b) to give the proportion of native collagen in the protein solution; and

wherein step (b) is carried out at a temperature less than the thermal melting temperature of the collagen being tested for.

35. A method according to claim 34 wherein the collagen being tested for is a cold water fish skin collagen.

36. A method according to claim 35 wherein step (b) is carried out at less than 20° C.

37. A kit for determining the amount of native collagen in a sample, the kit comprising copper sulphate, potassium sodium tartrate, and Sirius red.

38. A kit according to claim 37 wherein the tartrate and Sirius Red are in solution ready for use.

39. A kit according to claim 37 or claim 38 which further comprises a standard for the collagen being tested.

* * * * *

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(54) METHOD FOR ISOLATING SPONGE COLLAGEN AND PRODUCING NANOPARTICULATE COLLAGEN, AND THE USE THEREOF

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(57) ABSTRACT

The invention relates to a method for the simplified isolation in high yields of sponge collagen, especially from marine sponges, and to the production of collagen nanoparticles from collagen. The invention further relates to the use thereof for influencing cell-dependent processes in vitro and in vivo, especially when orally or topically administered to treat inflammatory, preferably cyclooxygenase-dependent diseases.

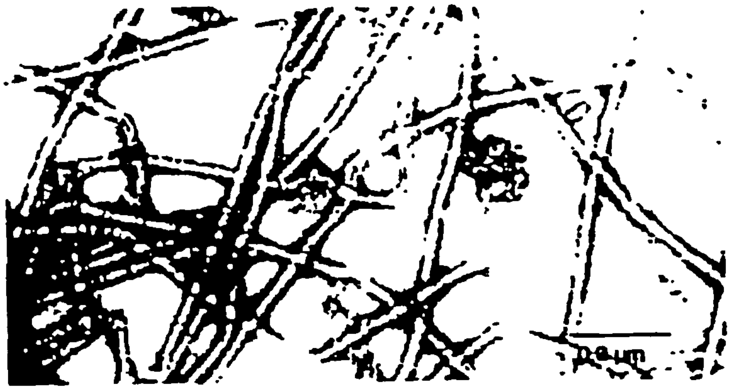

Figure 1

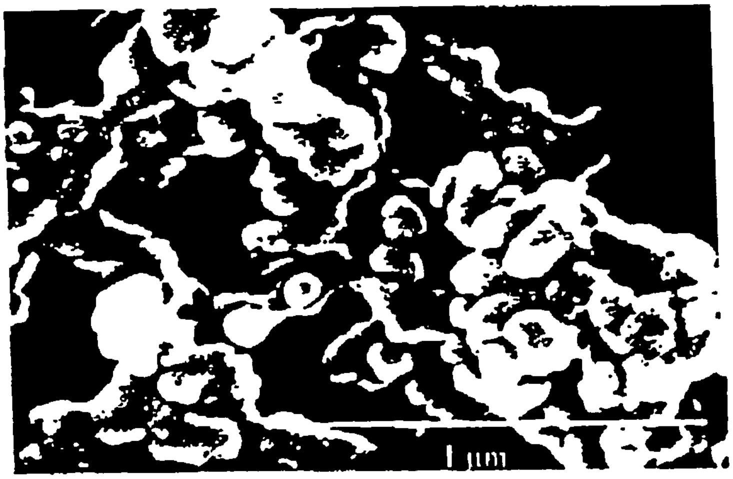

Figure 2

Size Distribution of Nano-Particles from Sponge Collagen made of Chondrosia reniformis

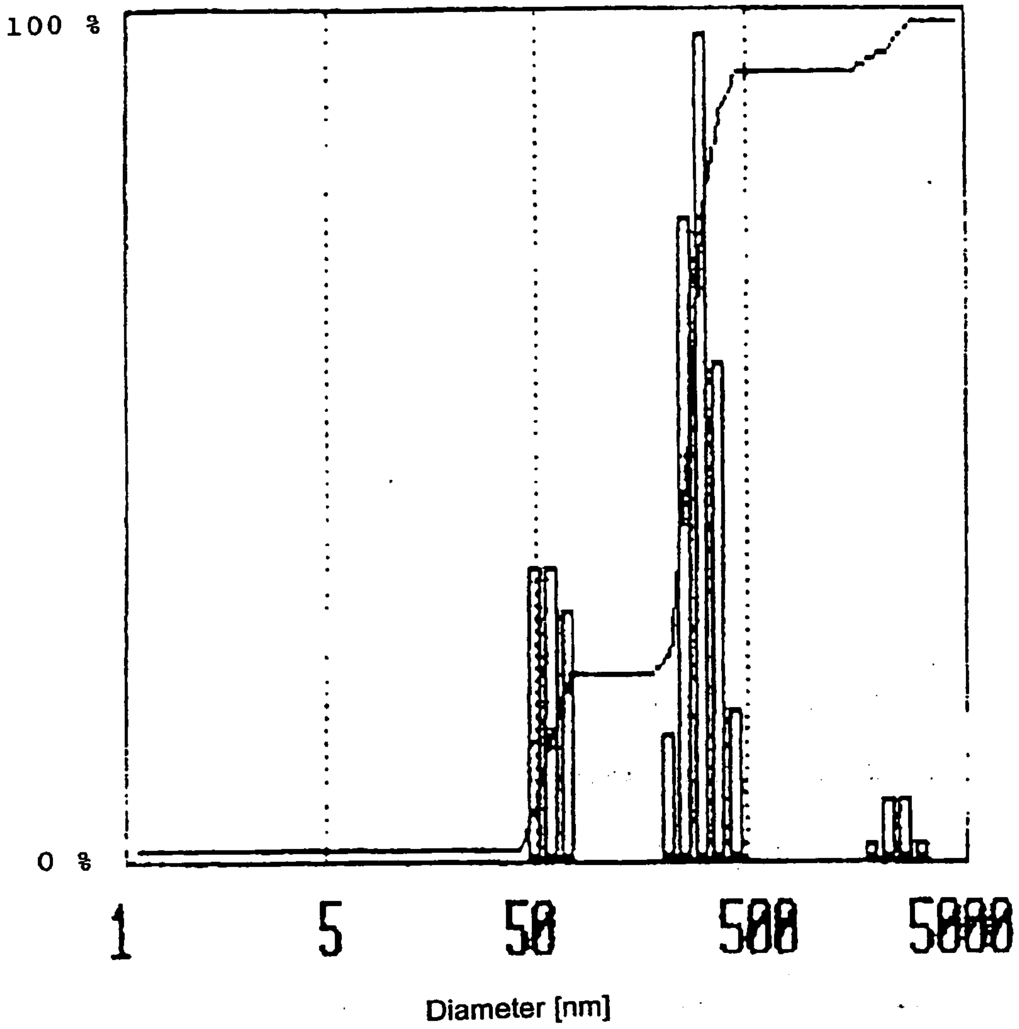

Fig. 3

SKIN PENETRATION THROUGH NANO-PARTICLES IN VITRO

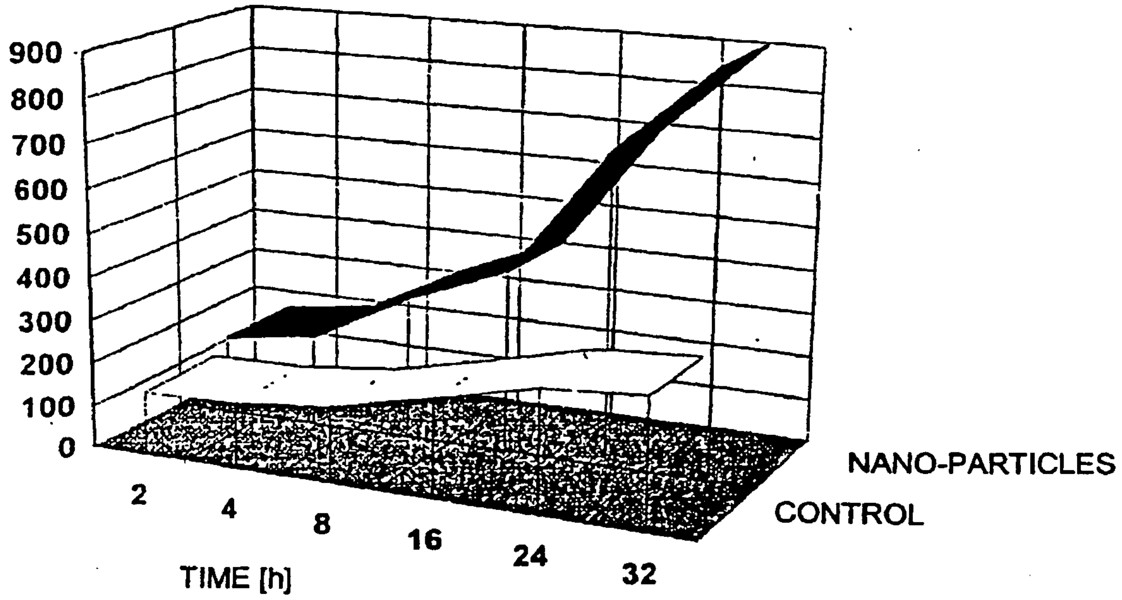

Fig4

Red (upper) curve: nano-particles

Yellow (lower) curve: control

METHOD FOR ISOLATING SPONGE COLLAGEN AND PRODUCING NANOPARTICULATE COLLAGEN, AND THE USE THEREOF

[0001] The present invention relates to the method described in the claims for a simplified and thus commercially usable isolation of sponge collagen, in particular from marine sponges, and especially sponges from the category of the Chondrosiidae, as well as for the production of collagen nanoparticles from collagen.

[0002] The invention furthermore relates to the use of such collagen for the production of a substance for influencing cell-dependent processes in vitro and in vivo. In particular the application relates to the use of such isolated collagen and/or of collagen nano- or microparticles for the preparation of creams, ointments, suspensions, tablets, capsules, also the delayed release, implants, band aids, foams etc. for covering wounds, active ingredient carriers in parenterals and enterals, eye drops, nano-capsules as active ingredient carriers and carriers for active ingredients through the skin and mucous membranes as well as vessels and organ membranes. The collagen produced pursuant to the invention is suited for the production of substances for external and internal, preferably oral and topical applications for the treatment of inflammatory, especially also cyclooxygenase-dependent diseases in vivo as well as furthermore for promoting the growth of permanent and neuronal cells in vitro.

[0003] Collagen is a bio-degradable and compatible protein and is used as the starting material for a broad range of applications in the pharmaceutical industry, in cosmetics and food chemistry.

[0004] So far collagen has been gained from animal skin and the bones of pigs, calves and cattle. Here the possibility of infecting the user with pathogenic germs, viruses and above all with BSE (bovine spongiforme encephalopathy) represents a decisive disadvantage.

[0005] Sponges are more than 600 million years old and thus represent the oldest members from the group of the metazoans. Like all multiple-cell animals, sponges contain the connective tissue protein collagen. Sponge collagen represents a water-insoluble protein, which has the typical amino acid composition of familiar collagen types. Glycine, for example, represents about every third amino acid, and the percentages of proline as well as hydroxy-proline are high.

[0006] Sponge collagen can be isolated with various methods. So far fresh material has been used for this with little practical relevance. The cleaning method for such isolated sponge collagen has been largely relatively complex from an industrial point of view (e.g. gel filtration). And the yield as well was not yet satisfactory. For example, from 1000 g sponge material, 21 g sponge collagen was isolated.

[0007] This is described by B. Diehl Seufert et al. in J. Cell Sc. 79, 271-85 (1985). The isolation of collagen from the *Geodia cydonium* and *Chondrosia renifornis* sponges with natural amino acid composition while avoiding denaturation reagents takes place through the suspension of homogenized fresh material in Tris-HCl buffer, pH adjustment to 9, centrifugation and homogenization. Cleaning occurs with gel filtration, wherein as mentioned above, 1.7 or 3.1% collagen is obtained.

[0008] There is a need therefore for a simple and economical method for isolating collagen from sponges, while simultaneously avoiding the disadvantages for collagen gained from animal starting material, and obtaining high yields.

[0009] Beyond that, it is particularly advantageous to produce the smallest particles of collagen possible.

[0010] The production of collagen micro-particles has been described so far for native calf collagen. However it was only possible to produce micro-particles in the range from 3 to 40 μm, e.g. as described in B. Rossler, J. Kreuter and D. Scherer 'Collagen Microparticles, Preparation and Properties', J. Microencapsulation, Vol. 12, No. 1, 49-57 (1995). This range however is not very suitable for pharmaceutical and cosmetics applications. The intravenous application is associated with the risk of embolism. The application on skin or on the eye slows down the release of attached substances so that it does not occur within a useful time frame before the preparation is washed off or is removed e.g. through drainage from the eye.

[0011] Additionally, the relatively large particle size can create an unpleasant scratchy sensation. For this reason it is necessary to restrict the range, i.e. keep it below 3 μm or smaller.

[0012] It is therefore the object of the present application to develop a product-oriented isolation method for sponge collagen, which leads to good yields of a product with a high collagen percentage in a simple and effective manner, wherein minimal toxicological risk should exist. At the same time, the starting material should be inexpensive and practice-relevant so that the addition of deep-frozen fresh material as starting material can be foregone (as it has been used until now).

[0013] Furthermore collagen particles are to be produced which have as small a size as possible, particularly which are in the micro and nano range.

[0014] This task is resolved with the invention in that the fresh starting sponge material, which does not have to be frozen, is placed in alcohol, subsequently washed with water, treated with an extractant, preferably with a pH value of 7-12, in particular 8-10, above all 9-95 or 9.5 and the resulting collagen extract is worked up. This takes place preferably by increasing the pH value of the suspension to a pH of 8-11, in particular 9-10, above all 9, stirring, centrifugation and subsequently through a reduction of the pH value of the residue, centrifugation and isolation of the precipitate.

[0015] This process eliminates another cleaning process, such as e.g. through gel filtration or the like. The cleaned collagen product, which can be freeze-dried for conservation purpose if desired, is then obtained in a high yield.

[0016] Suitable starting materials are the conventional, familiar, especially marine sponges such as, for example, Demospongiae [Rupert Riedel, Fauna und Flora des Mittelmeeres (Fauna and Flora of the Mediterranean Sea), Paul Parey Publishing Company, 1983], particularly such with high collagen percentage such as sponges of the category of the Geodiidae, Dysideidae, Spongiidae, Suberitidae, Oscarellidae, Axinellidae and others, above all from the group of the Chondrosiidae, which beyond that have minimal toxicological risk. Chondrosiidae or also Axinellidae are particularly preferred.

[0017] The familiar straight-chain or branched-chain aliphatic C_1-C_{15} alcohols such as ethanol, isopropanol, butanol, tert. butanol, pentanol, cyclohexanol, hexanol etc are suitable alcohols. Beyond that additives of aromatic alcohols such as thymol as well as benzyl alcohol and combinations thereof, e.g. among each other or with the aliphatic alcohols, in particular ethanol or isopropanol, are also possible.

[0018] C_1-C_4 aliphatic alcohols, and especially ethanol, are particularly preferred. The quantity of alcohol depends on the quantity of starting material since said material is placed in alcohol.

[0019] Suitable extractants are e.g. familiar systems such as Tris-HCl buffer (preferably containing 10 mM EDTA, 8M-urea, 100 mM-2-mercaptoethanol) or alkali-containing buffer systems, e.g. potassium carbonate-containing, phosphate-containing, nitrogen- or sulfate-containing buffers with a suitable pH value. In this case, the substance is used in excess in relation to the weight of the starting material that is used, especially at 2-8 times the weight quantity, especially 3-6 times and above all 5 times the quantity.

[0020] Such buffers are generally known and described for example in the European Pharmacopeia.

[0021] The pH value can be adjusted or reduced with known means. Suitable for this are e.g. buffer solutions such as the above-mentioned nitrogen, phosphate, sulfate, potassium carbonate-containing or organic buffers such as acetate or citrate containing buffer systems with suitable pH value.

[0022] Precipitation of the collagen from the residue after centrifugation can be accomplished through a pH value reduction, especially to values between 2 and 6, especially 5-3 and above all pH 4, through appropriate means such as organic acids, e.g. acetic acid (e.g. 100%), citric acid, ascorbic acid, propanoic acid, formic acid or lactic acid etc. or through diluted inorganic acids, e.g. hydrochloric acid, phosphoric acid, sulfuric acid and the like. The subsequent isolation of the precipitate can occur with renewed centrifugation, wherein preferably—as is also the case with the initial centrifugation—the temperature can be lowered, especially to values smaller than 10° C.-1° C., preferably 6° C.-1° C., especially 2° C.

[0023] The product that is obtained (collagen sediment) is pure and can be freeze-dried for conservation if desired.

[0024] FIG. 1 (see example 2) shows such a lyophilized product obtained pursuant to the method of the invention, wherein the periodic cross stripes of the collagen filtration that is typical for collagen can be recognized.

[0025] With the invented method it is thus possible to obtain in high yields pure collagen without complex cleaning steps.

[0026] Surprisingly, it has been shown that the typical features for collagen, such as periodic cross stripes of the fibrils, remains present and thus is natural.

[0027] The method is particularly suited for industrial 'scaling-up'. The cleaning of isolated sponge collagen could be simplified according to the invention in favor of a practice-oriented method. The yield of sponge collagen is very high at about 10-40%, or 30% on average. Since the sponge species utilized are safe from a toxicological point of view, especially e.g. when used for edible sponges from the families of the Chondrosiidae or Axinellidae, one can assume that the sponge collagen obtained this way is very compatible and largely safe for humans from a toxicological point of view. Beyond that, the isolated sponge collagen exhibits considerable advantages over pig, calf and cattle collagen with regard to the BSE problem.

[0028] As mentioned above it is beneficial to produce collagen material in particularly small particle sizes. So far native calf collagen has been emulsified and cross-linked with glutardialdehyde solution. The particles obtained had a size of 3-40 μm.

[0029] According to the invention now particle sizes of less than 3 μm can be achieved for the first time.

[0030] Collagen, obtained based on familiar methods from starting materials of various, e.g. animal origins as described above from calves, pigs, cattle or sponges, especially such obtained from sponges with the invented method, is dispersed e.g. in water and initially homogenized. The pH value can be adjusted preferably then or if necessary before the first homogenization to pH 7-11, especially 8-10, preferably 9-10, above all 9.5, and the substance can be homogenized again. Subsequently it is emulsified, through the addition of an emulsifying agent and a fatty phase such as e.g. through the addition of paraffin and Span ® or e.g. through other W/O emulsifying agents such as cetylstearyl alcohol, glycerin monostearate or Span ® 85 (Sorbitantrioleate), or similar products such as Arlacel 85, Span 65 (Sorbitantristearate), Arlacel 65, propylene glycol monostearate, sorbitan monooleate, Span 40, Arlacel 40, Span 20 or possible O/W emulsifying agents of the familiar kind (polyethylene glycols with suitable ethoxylation degree; such as e.g. with HLB value 8-13, e.g. polyoxyethylene glycol-400-monostearate, oleyl ether, monolaurate, sorbitan monolaureate, sorbitan tristearate) in a weight of more than 2 to 5 times the quantity of the collagen dispersion. Apart from paraffin, similar liquid hydrocarbons, such as e.g. isoparaffin, dioctylcyclohexane (Cetiiol® S), isohexadecane (Arlamol® HD), triglycerides such as Miglyol®, squalane or squalene or mixtures thereof are suited. The quantity of the fatty phase is specified together with the quantity of emulsifying agent and is, as mentioned above, 2-5 times the collagen quantity.

[0031] Subsequently the product is cross-linked with an excess, i.e. more than twice the amount than before, i.e. at least 2 to 6 times the weight, preferably 3-5 times, especially 4 times, in relation to the collagen quantity that is used, of cross-linking agents such as e.g. bi-functional acid chlorides, aldehydes, especially glutardialdehyde (e.g. a 25% aqueous solution thereof) or maleic acid dialdehyde or dichloride.

[0032] The reaction is worked up in a suitable fashion, initially interrupted e.g. through the addition of H_2O_2. For this, a considerably higher weight is used than previously, i.e. 2-6 times, preferably 3-5 times, especially 4 times the quantity in relation to the collagen quantity that is used. Alternatively, other familiar suitable oxidation agents such as HNO_3 can also be used to terminate the process. Reconditioning occurs e.g. through centrifugation, suspension of the sediment in alcohol, e.g. isopropanol, renewed centrifugation and additional runs through this cycle. In the last cycle, suspension with a weak acid occurs, e.g. ascorbic acid or another organic acid, e.g. citric acid, lactic acid, formic acid and the like. This sediment is then heated, e.g. to temperatures >50° C., especially to 75° C., stirred again for several hours (e.g. 24), centrifuged and rinsed with water.

[0033] The material that is obtained can be freeze-dried. **FIG. 3** depicts the size distribution of nano-particles, which were produced as described in the subsequent example 3. This shows that more than 95% of all particles are nano-particles.

[0034] Surprisingly the smallest particulate collagen can be produced with the above-described method when the starting material, which is suitably dispersed e.g. in distilled water, is initially homogenized before emulsification and cross-linkage. Homogenization suitably occurs through ultrasound treatment, Ultraturrax or high pressure homogenizer or micro-fluidizer. After adjusting the pH value e.g. with potassium carbonate from pH 4 to pH 8, preferably another homogenization process can be conducted. Cross-linkage occurs with a larger quantity of cross-linking agents than has been used until now.

[0035] Collagen is particularly preferred as the starting material, obtained pursuant to the above-described isolation method of the invention from marine sponges of the family of the Chondrosiidae.

[0036] This new method for producing collagen micro-particles from collagen, especially from sponge collagen such as e.g. from marine sponges of the family of the Chondrosiidae, consequently makes the production of particles in a size range from 150 nm to 3 μm possible, in contrast to familiar methods for producing collagen, which enable micro-particles of only 3-40 μm.

[0037] The collagen and nano-particulate collagen obtained in the manner of the invention find broad applications in pharmacy, medicine, cosmetics and food chemistry such as creams, ointments, for applications on skin and mucous membranes, suspensions, tablets, capsules, also with delayed release, implants, band aids, foams, sponges, fleece and the like for covering wounds, active ingredient carrier in parenterals and enterals, eye drops, nano-capsules as active ingredient carrier and carrier for active ingredients through the skin and mucous membranes as well as vessels and organ membranes. These substances were prepared in the familiar fashion. The production methods for this are e.g. described in DAB and are known both for the manufacture of ointments, creams as well as of tablets, etc. wherein conventional quantities are used.

[0038] Surprisingly it has been shown that the collagen prepared pursuant to the invention, especially the nano-particulate collagen, exhibits an anti-inflammatory effect especially in the case of topical and also oral application apart from intravenous or intraperitoneal application. It turns out that the substance is an inhibitor of cyclooxygenase and also has an anti-oxidative effect. Particularly surprising is the anti-inflammatory effect e.g. for joint problems, arthritis and especially analogous diseases of the locomotor system, in particular when the substance is administered orally. In this case, the above-described, especially marine sponge collagen is suspended as such or in the freeze-dried state in a suitable beverage, such as water or juices, and administered as a beverage, e.g. in quantities of 2-10 g, especially 2-6 and preferably 3-5 g/day. It can be available as a solid as well as a powder or granulated collagen as described below, preferably in a quantity of 1-10, especially 2-8 and above all 3-5 g/250 ml of fluid, preferably water.

[0039] As a substance which can be topically applied, both a penetration reinforcement of another active substance and/or in combination therewith, such as, for example, of a vitamin (vitamin A, C, E or their mixtures) or other topically active substances for the treatment of the skin such as avarol, avarone or plant extracts, such as Extr. Cepae or Extr. *Echinaceae pallidae*, as well as in particular an anti-inflammatory effect can be observed. The described collagen is especially suited for the treatment of skin changes, e.g. of degenerative type, or when the skin is damaged due to outer or inner influences, e.g. for the treatment of erythema after sunburns, UV radiation or injuries or psoriasis. The substance is also suitable for the indications of this type mentioned below. The topical agent can be available in the form of creams, ointments, lotions on a familiar basis, or especially in the form of a gel such as a hydrogel e.g. on the basis of polyacrylate or an oleogel e.g. made of water and Eucerin.

[0040] The employed oleogels made of an aqueous and a fatty phase are based particularly on *Eucerinum anhydricum*, a basis of wool wax alcohols and paraffin, wherein the percentage of water and the basis can vary. Furthermore additional lipophilic components for influencing the consistency can be added, e.g. glycerin, polyethylene glycols of different chain length, e.g. PEG400, plant oils such as almond oil, liquid paraffin, neutral oil and the like. Such prescriptions are generally known and described in DAB10, or in the European Pharmacopeia, current edition, e.g. 2000. The hydrogels can be produced through the use of gel-forming agents and water, wherein the first are selected especially from natural products such as cellulose derivatives, such as cellulose ester and ether, e.g. hydroxyethyl-hydroxypropyl derivatives, e.g. tylose, or also from synthetic products such as polyacrylic acid derivatives, such as Carbopol or Carbomer, e.g. P934, P940, P941. They can be produced or polymerized based on known regulations, which are described in current pharmacopoeias such as e.g. DAB10 or the European Pharmacopeia, current edition, e.g. 2000, from alcoholic suspensions by adding bases for gel formation.

[0041] Per 100 g of gel, the gels comprise 0.01-30 g, especially 0.01-10 g and above all 0.01-8 g and preferably 0.1-5 g collagen, i.e. accordingly 0.01-30; 0.01-10; 0.01-8; 0.1-5%.

[0042] This way a substance can be made available, which does not exhibit the side effects of existing substances used for the treatment of inflammatory diseases, such as e.g. NSDAIs, and are above all orally well tolerated.

[0043] The above-described agents are therefore suited for compositions for the treatment of inflammations in a living being and for the treatment of other inflammation-related malfunctions. Examples for inflammations and inflammation-similar malfunctions are arthritis, including rheumatoid arthritis, spinal joint problems, gout, systemic lupus erythematosis, osteoarthritis and juvenile arthritis, furthermore asthma, bronchitis, menstrual cramps, tendonitis, bursitis and conditions associated with the skin such as psoriasis, excema, burns and dermatitis. The described agents are also suitable for the treatment of gastrointestinal conditions, such as inflammatory intestinal disorder, Crohn's disease, gastritis, irritable bowel syndrome and ulcerative colitis, for the treatment of inflammation in diseases, such as vascular illnesses, periarteritis, Hodgkin's disease, sclerodema, rheumatoid fever, type I diabetes, myasthenia gravis, sarcoidosis, nephrotic syndrome, Behcet's syndrome, polymyositis, bleeding gums, hypersensitivity, conjunctivitis, swelling occurring after injuries, myocardialischemia etc.

[0044] Additionally this invention comprises a category of compositions, containing a collagen produced as described, especially marine sponge collagen, in particular nano-particulate collagen, together with one or more non-toxic pharmaceutically tolerable vehicles and/or diluting agents and/or adjuvants (described in the following under the collective term "vehicle" materials) and possibly other active ingredients. The substances pursuant to the invention can be administered as mentioned above in any suitable way and at a dosage that is effective for the intended treatment. The substance can, for example, be administered intravascularly, intraperitoneally, subcutaneously, intramuscularly, especially orally or topically.

[0045] For oral administration, the agent can assume the form of e.g. a tablet, capsule or suspension. In this case the substance is preferably produced in the form of one dosage unit. The substance can additionally be administered through injection as a composition, wherein for example saline solution, dextrose or water can be used as a suitable vehicle.

[0046] The quantity of the administered substance and the dosage schedule for the treatment of an illness with the described substance depend on a multitude of factors, including the age, weight, sex and medical condition of the patient, the severity of the illness, the administration route and the frequency of administration, as well as on the compound in question used, and can therefore fluctuate greatly. The substances of the invention can generally be combined with one or more adjuvants, which are suitable for the specified administration route. If administration occurs per os, the described collagen product can also be mixed with lactose, saccharose, starch powder, cellulose ester or alkane acids, cellulose alkyl ester, talcum, stearic acid, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulfonic acids, gelatins, gum arabic, sodium alginate, glycols, polyvinyl pyrrolidone and/or polyvinyl alcohol and then be put in tablets or capsules for easy administration. Such capsules or tablets can contain a controlled release formulation, as can be provided in a dispersion of the substance in hydroxypropyl methyl cellulose. Formulations for parenteral administration can exist in the form of aqueous or non-aqueous isotonic sterile injection solutions or suspensions. These solutions and suspensions can be produced from sterile powders or granules with one or more vehicles or diluents, as they were mentioned for the use in formulations for oral applications. The agents can be dissolved in water, polyethylene glycol, propylene glycol, ethanol, corn oil, cotton seed oil, peanut oil, sesame oil, benzyl alcohol, sodium chloride and/or various buffers. Other adjuvants and types of administrations are known.

[0047] The above-described collagen is used in a cell suspension in a quantity of 0.01-150 μg/ml, especially 0.1-100 μg/ml and above all 1-60 μg/ml of cell suspension as a means for influencing cell-dependent processes in vitro. Permanent cells such as CEM cells or neuronal cells like cortical cells are particularly suited as cells to promote growth. Suitable collagen is either the directly isolated product as described above or the nano-particulate product of the invention in accordance with the present application. The collagen is then added either in a solid state to the cell sample and is available as suspension, or is dissolved in an alkaline aqueous medium (pH value greater than 7, especially greater than 8), placed into the sample container, acidified until the collagen precipitates, and then the cell sample is added.

[0048] The invention is explained in more detail based on the following examples.

EXAMPLE 1

Method for Isolating Sponge Collagen from Marine Sponge of the Family of Chondrosiidae

[0049] The sponge material is collected, broken down into coarse pieces and immediately placed in ethanol for conservation. The sponge pieces are washed three times with distilled water and homogenized with a mixer. The broken down material is mixed with five times the weight of an extractant (pH 9-5; 10 mM-EDTA, 8 M-urea, 100 mM-2-mercaptoethanol). The pH value of the resulting dark brown suspension is adjusted with tris from pH 7 to pH 9. This suspension is stirred for 24 hrs at room temperature and subsequently centrifuged (5000 g, 6 min., 2° C.). The pellet is discarded. The pH value of the residue is adjusted to pH 4 with 100% acetic acid. A precipitate forms, which is collected (20,000 g, 20 min., 2° C.). The pellet is mixed with water and centrifuged again (20,000 g, 10 min., 2° C.). This results in a clear, slightly colored residue as well as the cleaned collagen sediment, which can be lyophilized for conservation purposes.

[0050] The collagen yield is around 30% (freeze-dried collagen sediment in relation to freeze-dried sponge material).

EXAMPLE 2

Electron-microscopic Characterization of the Isolated Sponge Collagen (TEM/Transmission Electron Microscopy)

[0051] For examination purposes, the freeze-dried collagen sample is negatively contrasted with a 2% phosphotungstic acid based on the so-called single droplet method (see J. R. Harris, Negative staining and cryoelectron microscopy. The thin film techniques, Royal Microscopial Society Microscopy Handbook No. 35. BIOS Scientific Publishers Ltd., Oxford, UK).

[0052] In the enlargement, one will recognize the periodic cross stripes of the collagen fibrils, which are typical for collagen, in the TEM image in accordance with **FIG. 1**.

EXAMPLE 3

Preparation of Collagen Micro-particles from Sponge Collagen of the Family of Chondrosiidae

[0053] Initially a 0.75% sponge collagen dispersion is produced by mixing the freeze-dried material with distilled water and homogenizing it with an Ultra-Turrax. The pH value of the dispersion is adjusted to pH 9.5 with potassium carbonate. Subsequently the dispersion is homogenized a second time with the Ultraturrax.

[0054] A mixture is prepared from 10 g Span® and 250 g liquid paraffin, to which 85 ml of the 0.75% collagen dispersion is added. This mixture is emulsified for 10 minutes at about 6000 rpm with an Ultraturrax (Ika Factory, Staufen, Germany). Subsequently, the emulsion is stirred constantly with wing stirrer. For the purpose of cross-linkage of the collagen chains, 12 g of a 25% aqueous glutardialdehyde solution is added. This reaction is completed after 12 minutes through the addition of 16 g of a 30% aqueous hydrogen peroxide solution. The preparation is stirred for an additional 15 minutes. The emulsion is diluted with 100 ml 2-propanol.

[0055] For cleaning the CMPs, the emulsion is centrifuged (30 minutes/10444 g), the oily residue is discarded and the sediment is suspended in 50% 2-propanol. Subsequently the suspension is centrifuged again. This cleaning step is repeated. The CMP sediment that is obtained is suspended in a 4% aqueous ascorbic acid solution. This suspension is heated (75° C., 30 minutes) so as to destroy residual quantities of oxidizing substances. The mixture is stirred for 24 hrs on a magnetic stirrer at room temperature. The CMP is subsequently cleaned through centrifugation (30 minutes, 10444 g) and then washed twice with water.

[0056] The material is freeze-dried. The CMP yield is around 10%.

EXAMPLE 4

Scanning Electron Microscopic Examination of the Collagen Micro-particles Pursuant to Example 3

[0057] The collagen micro-particles were coated with about a 4 nm thick platinum film and examined with the SE 4500 Hitachi S scanning electron microscope. The size of the spherical collagen particles lies at 120 to 300 nm (**FIG. 2**).

EXAMPLE 5

Particle Size Determination/Photon Correlation Spectroscopy (PCS) of Collagen Particles Pursuant to Example 3

[0058] The particle size determination process was conducted with the help of photon correlation spectroscopy (PCS). The lyophilized sponge collagen was resuspended in filtered water (pH 9.8, adjusted with K_2CO_3) before measurement. This resulted in a particle size distribution of 150 nm to 3 μm. While the lower limit agrees quite well with the results of the scanning electron microscopy, at 3 μm the upper range is clearly above the value of 300 nm. This could be due to agglomerates, which form in water during redispersion and cannot be dissolved even in the ultrasonic bath.

[0059] **FIG. 3** shows the size distribution of the nano-particles produced pursuant to example 3 and shown in **FIG. 2**. As can be seen, more than 95% of all particles are nano-particles. 25% of all particles have an average diameter of 50 nm and are more than 70% in the range of 300 nm (abscissa=diameter of particles [nm], ordinate=parts in %).

EXAMPLE 6

Thio-barbituric Acid (TBA) Antioxidant Test with Collagen from *Chondrosia reniformis*

[0060] The test is based on the formation of a reaction product, measurable at 532 nm, of thio-barbituric acid (TBA) with malondialdehyde (MDA), which arises among other things in the oxidative decomposition of multiple unsaturated fatty acids, as they are contained in trilinolenin (Sigma).

[0061] Trilinolenin and the test substances, sponge collagen produced from *Chondrosia reniformis* as described in example 1, and butylhydroxytoluene [BHT] are dissolved in DMSO.

[0062] 400 μl of trilinolenin are incubated in 2 ml HAM F10—Medium for 4 hours at 37° C. in the presence of DMSO as control, or DMSO-containing substance solutions.

[0063] For determining the overall TBA-reactive material, 1.0 ml of the incubation batch is removed and mixed with 1.5 ml of TBA solution (0.67% in 0.05 M NaOH) as well as with 1.5 ml of tri-chloracetic acid (20%).

[0064] After a 60-minute incubation in a boiling water bath the samples are cooled down and centrifuged for 10 minutes at 2000 rpm. The visual density is determined at 532 nm. Fresh tetramethoxypropane, which is obtained under the test conditions from MDA, serves as calibration standard. The results are calculated as μmol MDA equivalent. Triple determination processes are conducted.

[0065] Table 1: effect of sponge collagen in thio-barbituric acid (TBA)—antioxidant test (% inhibition (±SD) of the formation of TBA reactive material compared to BHT as standard substance)

TABLE 1

	Concentration [μM]			
Substance	5	10	20	50
BHT	71 (±1.5)	73 (±2.1)	79 (±0.3)	85 (±0.5)
Collagen*	18 (±1.3)	37 (±2.3)	41 (±3.9)	47 (±1.8)

*collagen = collagen from *Chondrosia reniformis*

[0066] Surprisingly, collagen from *Chondrosia reniformis* inhibits the formation of TBA-reactive material. This was not known until now. The potency is about 50% of the standard BHT substance. Collagen from *Chondrosia reniformis* shows medium effects with a flat dosage effect relation curve in the concentration range that was examined.

EXAMPLE 7

In vitro Inhibition of Cyclooxygenase

[0067] The cyclooxygenase activity was determined in monocytes in the presence or absence of the agonist lipopolysaccharide [LPS] (L-4130; Sigma, St. Louis). For this purpose, human monocytes were kept in a density of 106 cells per ml in RPMI-1640 medium for 24 hrs. Subsequently 30 μM arachidonic acid was added to the cultures and the enzyme activity was measured 30 minutes later based on the production rate of prostaglandin E2.

[0068] Table 2:

[0069] Effect of sponge collagen, produced as described in example 1, on the cyclooxygenase activity in human monocytes.

[0070] The values represent mean values (±S.D.) of five independent results.

TABLE 2

Addition of LPS	Collagen (μg/ml)	Cyclooxygenase Activity (pg PGE2/106 cells)
none	0	88 ± 10
	1	71 ± 9
	5	52 ± 6
	0	243 ± 22
	0.5	187 ± 19
	1.0	79 ± 7
	5.0	61 ± 7

[0071] Result:

[0072] After the addition of LPS, an increase in the cyclooxygenase activity occurs from 88 to 243 pg PGE 2 per 106 cells within 30 minutes. Increasing concentrations of sponge collagen from *Chondrosia reniformis* eliminate the stimulating effect of LPS on the cyclooxygenase. At a concentration of 3 μg/ml, the induction of the cyclooxygenase is reduced to 61%.

EXAMPLE 8

Sponge Collagen for Chronic-inflammatory and Degenerative Motion Problems

[0073] The *Chondrosia reniformis* sponge is being eaten still today. 10 patients with chronic arthritis therefore agreed to an oral treatment. 5 g collagen from *Chondrosia reniformis*, produced as described in example 1, is to be taken suspended in a beverage three times a day for a period of 4 weeks. All patients unanimously reported, after a short period of time, decreased pain in the joints and improved motion.

EXAMPLE 9

Penetration of the Skin of Naked Mice in vitro

[0074] Nano-particles gained from the *Chondrosia reniformis* collagen, produced as described in example 3, were loaded with 14C-marked retinol (charge rate 15%) and worked into an oleogel made of water/Eucerin as described above (collagen: 1%, added as 10% suspension).

[0075] Diffusion cells according to Franz [n=6] were filled with physiological saline solution and the acceptor cells were covered with a freshly prepared section of the skin of naked mice. 100 mg of the gel loaded with 14C-marked retinal was applied onto the skin sections. Appropriate gels with 14C-marked dissolved retinal served as controls [n=6].

[0076] After 2, 4, 8, 16, 24 and 36 hours the 14C activity in the acceptor cell was determined.

[0077] The results are shown in **FIG. 4** and clearly prove the superiority of sponge collagen nano-particles in the penetration of mice skin compared to a hydrogel, which contains the marked substance only in dissolved form.

EXAMPLE 10

Examination on UV or Heat-induced Erythema of Human Skin

[0078] The examinations were conducted on volunteers, who suffered acutely from sunburns [n=7] or burns [n=3]. Sponge collagen nano-particles [1%] in accordance with example 3 were topically applied in a polyacrylate gel, produced as described above (1% collagen, added as 10% suspension).

[0079] The clinical effect of the applied gel on the UV or burn-related change in skin was evaluated macroscopically 1 hour later after a large scale application of the gel as well as 2, 4, 6 and 24 hours later, and the patient was questioned about his/her condition.

[0080] Result:

[0081] Sponge collagen reduced the erythema quickly during the first few hours after the erythema-triggering event. The one-time application had a long-term inhibiting effect of up to 5 hours. This could be reinforced by incorporated familiar topical inflammation-inhibiting substances into the nano-particles, such as vitamin E, Avarol, Avarone or plant extracts, such as extracted Cepae or extracted *Echinaceae pallidae*.

EXAMPLE 11

Toxicity Examinations on the Collagen Pursuant to the Invention

[0082] In order to determine toxicity, the influence of sponge collagen onto cellular growth in cultures was examined on a permanent cell line and on freshly prepared neuronal cells.

[0083] 1. CEM Cells (Permanent Human Leukemia Cells)

[0084] CEM cells [Sechoy et al., Exp. Cell Res. 185: 122, 1989 and Avramis et al., AIDS 3: 417, 1989] were either not treated with sponge collagen (control sample) or incubated with various concentrations thereof.

[0085] Cell growth was the examination parameter. CEM cells were used at a concentration of $0.2\text{x}10^6$ cells/ml culture medium inoculation of the culture. After a 4-day incubation, the density of the CEM cells was $1.9\text{x}10^6$ cells/ml. This value forms the control value. After an additional 4 days the cell density was determined again.

[0086] 2. Neuronal Cells

[0087] Cortical cells (neurons) were prepared from the brains of newborn Wistar rats and kept under cell culture conditions. The culture medium used was MEM medium (with 10% horse serum), and it was incubated at 90% humidity and 10% CO_2 atmosphere. Generally after 48 hours of incubation in culture, the cells were used for the experiments. The culture contain neurons in an overwhelming concentration (more than 70%) and about 20% GFAP positive astrocytes (Müller et al.: Europ. J. Pharmacol.—Molec. Pharmacol. Sect. 226: 209-214,1992). After an additional 4 days, the cell density was measured experimentally. The results are shown in table 3.

TABLE 3

	Concentration of the Sponge Collagen (μg/ml)	Cell Concentration × 1,000,000/ml
Control	0	1.9 ± 0.24
1. CEM Cells	0.1	2.0 ± 0.2
	1.0	2.5 ± 0.3
	10.0	2.9 ± 0.3
	100.0	2.7 ± 0.2
2. Neuronal Cells	0.1	2.3 ± 0.2
	1.0	2.7 ± 0.3
	10.0	3.4 ± 0.2
	100.0	2.5 ± 0.2

[0088] The results of the table represent mean values of 6 parallel experiments with standard deviations. The significance was determined with the Student t-test (Sachs, L.: Angewandte Statistik [Applied Statistics], Berlin: Springer-Verlag, pp. 209-216; 1984).

[0089] It is obvious that sponge collagen has no negative effects on the growth rate of permanent cells and neuronal cells in a primary cell culture. On the other hand, in the presence of sponge collagen, CEM cells and neuronal cells increase their growth rates significantly above their control values ($p<0.001$). It is only at concentrations around 100 μg/ml that no additional growth increase takes place.

[0090] This clearly shows that the collagen of the invention on one hand has an inhibiting effect on monocyte-dependent cells and enzymes deducible thereof such as cyclooxygenase for example, without being toxic, and on the other hand has a growth-enhancing effect on permanent cells and neuronal cells. Thus the collagen that is produced pursuant to the invention can be used not only for the in vivo treatment especially of cyclooxygenase-dependent illnesses, but above all also preferably for the in vitro influencing of permanent or neuronal cells. In these cases it can be employed in the above-mentioned quantities, especially between 0.01, preferably 0.1, and 100 μg/ml cell suspension.

1.) Method for isolating sponge collagen, characterized in that the starting material is placed in alcohol, subsequently washed, treated with an extractant, and that the collagen extract obtained this way is then reprocessed.

2.) Method pursuant to claim 1, characterized in that sponge collagen from marine sponges is isolated.

3.) Method pursuant to claim 1, characterized in that sponge collagen from Demospongiae, preferably Chondrosiidae, is isolated.

4.) Method pursuant to one of the claims 1-3, characterized in that ethanol is used as the alcohol.

5.) Method pursuant to one of the claims 1-4, characterized in that base buffer systems, preferably Tris buffers, are used as extractant.

6.) Method pursuant to one of the claims 1-5, characterized in that the extractant is used in excess amounts in relation to the weight of sponge starting material.

7.) Method pursuant to one of the claims 1-6, characterized in that, for the purpose of reprocessing, the pH value of the collagen extract is increased, the suspension is stirred, centrifuged, the residue is acidified and the precipitate is isolated.

8.) Method pursuant to one of the claims **1-7**, characterized in that the isolated collagen product is freeze-dried.

9.) Usage of a collagen product, manufactured pursuant to the method in accordance with one of the claims **1-8**, for producing a cosmetic, medical or pharmaceutical substance for topical, intravenous, intramuscular or oral applications.

10.) Method for producing nano-particulate collagen, characterized in that the starting collagen material is dispersed and homogenized, subsequently emulsified and cross-linked with an excess amount of cross-linking agent, and subsequently reprocessed.

11.) Method pursuant to claim 10, characterized in that a collagen product, as obtained pursuant to the method in accordance with one of the claims **1-8**, is used as the starting material.

12.) Method pursuant to claim 11, characterized in that collagen obtained from sponges of the category of Chondrosiidae pursuant to the method in accordance with one of the claims **1-8** is used.

13.) Method pursuant to one of the claims **10-12**, characterized in that collagen with a particle size of 150 nm to 3 μm is produced.

14.) Method pursuant to one of the claims **10-13**, characterized in that glutardialdehyde is used as the cross-linking agent.

15.) Usage of a collagen product, produced pursuant to a method in accordance with one of the claims **1-14**, for the production of a substance for influencing cell-dependent processes in vitro and in vivo.

16.) Usage pursuant to claim 15 for the production of a cosmetic, medical or pharmaceutical substance for topical, intravenous, intramuscular or oral applications in in-vivo-dependent processes.

17.) Usage pursuant to claim 16 for the production of a substance for treating cyclooxygenase-dependent illnesses.

18.) Usage pursuant to claim 16 for the production of an orally administered substance for treating illnesses of the locomotor system.

19.) Usage pursuant to one of the claims **15-18** for the production of a topically administered agent in the form of an oleogel or hydrogel.

20.) Usage pursuant to claim 15 as biochemical substance for in vitro growth-enhancing application in permanent and neuronal cells.

21). Method for in vitro growth enhancement of permanent and neuronal cells, characterized in that collagen, obtained pursuant to the method in accordance with one of the claims **1-8** or **10-14**, is added to the cell suspension at a quantity of 0.1-100 μg/ml suspension.

22.) Gel in the form of oleogel or a hydrogel, containing water, a gel basis as well as collagen, produced pursuant to the method in accordance with one of the claims **1-14**.

23.) Collagen suspension, containing collagen, produced pursuant to the method in accordance with one of the claims **1-14** in water at a quantity of 1-10 g collagen/250 ml.

24.) Collagen, obtained pursuant to the method in accordance with one of the claims **1-8** or **10-14**.

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(54) Title: METHOD FOR ISOLATING SPONGE COLLAGEN AND PRODUCING NANOPARTICULATE COLLAGEN, AND THE USE THEREOF

(54) Bezeichnung: VERFAHREN ZUR ISOLIERUNG VON SCHWAMMKOLLAGEN SOWIE HERSTELLUNG VON NANOPARTIKULÄREM KOLLAGEN UND DESSEN VERWENDUNG

(57) Abstract: The invention relates to a method for the simplified isolation in high yields of sponge collagen, especially from marine sponges, and to the production of collagen nanoparticles from collagen. The invention further relates to the use thereof for influencing cell-dependent processes in vitro and in vivo, especially when orally or topically administered to treat inflammatory, preferably cyclooxygenase-dependent diseases.

(57) Zusammenfassung: Die vorliegende Anmeldung betrifft ein Verfahren zur vereinfachten Isolierung von Schwammkollagen, insbesondere aus marinen Schwämmen, in hohen Ausbeuten sowie die Herstellung von Kollagennanopartikeln aus Kollagen, sowie deren Verwendung bei der Beeinflussung zellabhängiger Vorgänge in-vitro und in-vivo, insbesondere bei oraler oder topischer Verabreichung zur Behandlung entzündlicher, vorzugsweise Cyclooxygenaseabhängiger Erkrankungen.

ZA ZGODNOŚĆ
mgr inż. Jacek Czabajski
Rzecznik Patentowy

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